JOURNAL OF THE KENTUCKY ACADEMY OF SCIENCE

Official Publication of the Academy

Volume 67

Number 2

Fall 2006

All manuscripts and correspondence concerning manuscripts should be addressed to the Editor

The JOURNAL is indexed in BIOSIS, Cambridge Scientific Abstracts, Selected Water Resource Abstracts, State Academies of Science Abstracts, and Zoological Record.

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The JOURNAL is issued semiannually in spring and fall. Two numbers comprise a volume.

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Two chesnut fruit on an understory *Castanea dentata* growing in the Hi Lewis Pine Barrens State Nature Preserve on Pine Mountain, Kentucky. Photograph by Tracy S. Hawkins, USDA Forest Service. See article on page 73 of this issue.

JOURNAL OF THE KENTUCKY ACADEMY OF SCIENCE
ISSN 1098-7096
Continuation of
Transactions of the Kentucky Academy of Science
Volume 67 Fall 2006 Number 2

J. Ky. Acad. Sci. 67(2):73–80. 2006.

A Forest Transect of Pine Mountain, Kentucky: Changes Since E. Lucy Braun and Chestnut Blight

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ABSTRACT

In 1997, forest composition and structure were determined for Hi Lewis Pine Barrens State Nature Preserve, a 68-ha tract on the south slope of Pine Mountain, Harlan County, Kentucky. Data collected from 28 0.04-ha plots were used to delineate forest types. Percent canopy compositions were compared with those reported by Dr. E. Lucy Braun prior to the peak of chestnut blight. Four forest types were identified: *Liriodendron-Acer, Quercus-Tsuga,* Mixed *Quercus,* and *Pinus-Quercus.* Post-blight, little change has occurred in the *Pinus-Quercus* forest type on ridges and SSE aspects. On more mesic aspects, canopy gaps created by chestnut death were filled primarily by existing canopy species (*Quercus* spp.) and to a lesser degree by non-canopy species. Near the crest of the mountain, *Acer rubrum* has replaced *Castanea dentata* and assumed secondary importance to *Liriodendron tulipifera. Castanea dentata* remains an important component in the subcanopy of the four forest types and is present in the groundcover in three types. Except for the absence of *C. dentata,* species composition of Braun's forest types has remained relatively unchanged during the past 70 years; however, loss of *C. dentata* initiated changes in the relative importance of these species resulting in varying degrees of transition to post-blight forest types. Contribution of existing canopy species to importance values for the subcanopy and woody groundcover strata is less than that of fire-sensitive species, suggesting future changes in these post-blight forest communities.

KEY WORDS: Lucy Braun, *Castanea dentata,* chestnut blight, forest types, Pine Mountain

INTRODUCTION

Prior to the 20th century, American chestnut [*Castanea dentata* (Marsh.) Borkh.] was a dominant or codominant species of many hardwood forests of the eastern United States (Braun 1942, 1950; Gravatt 1949; Stephenson 1986; Schwadron 1995) and contributed up to 84.6% to the canopy composition in these mixed mesophytic forests (Braun 1942). However, introduction of chestnut blight [*Cryphonectria parasitica* (Murr.) Barr] to the United States in the early 1990's (Merkel 1905; Shelford 1963; Anagnostakis 1987) precipitated forest changes as *C. dentata* died out. In some forests, canopy gaps created from *C. dentata* death were filled by existing codominant species, while in others, subordinate species invaded the canopy (Braun 1950; Keever 1953; Woods and Shanks 1959; Good 1968).

Dr. E. Lucy Braun (1935) provided perhaps the best qualitative and quantitative information for pre-blight forests of the Cumberland Plateau in the Appalachian Plateaus Physiographic Province (Fenneman 1938) of Kentucky. She considered the extreme southeastern portion of Kentucky the geographic center of her "Mixed Mesophytic Forest Region" (Martin 1992). At the time of her research, *C. dentata,* although dying, was still present in

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the forest strata; therefore, she was able to include the species in her assessment of forest communities. The descriptions of Pine Mountain, in southeastern Kentucky, included percent canopy composition relative to topography and edaphic factors, as well as qualitative descriptions of lower forest strata and herbaceous groundcover. Further, Braun's (1935) study identified four complex forest associations on Pine Mountain that she further subdivided into sixteen forest types. *Castanea dentata* was present in all forest types, was a dominant canopy species in eight, and was described as "important" in two.

Chestnut persistence and replacement have been well documented for forests in Braun's (1950) Oak-Chestnut Forest Region (Keever 1953; Woods and Shanks 1959; Good 1968; Christensen 1977; Karban 1978; Arends 1981; Johnson and Ware 1982; Stephenson 1986; Stephenson et al. 1991; Hannah 1993; Parker et al. 1993). Braun's (1950) Beech-Maple (Schwadron 1995) and Western Mesophytic (Schibig et al. 2003; Myers et al. 2004) Forest Regions have received attention, as well. However, post-blight forest succession has gone largely undescribed in the Mixed Mesophytic Forest Region. The purposes of this study were to 1) delineate forest types and determine forest structure for a section of the southeastern dip slope of Pine Mountain, 2) compare post-blight canopy species percent composition with pre-blight values reported by Braun (1935), and 3) describe chestnut persistence and/or replacement in forest types on Pine Mountain during the post-chestnut blight era.

THE STUDY AREA

Pine Mountain is a prominent, monoclinal ridge extending approximately 200 km in a northeast-southwest direction, from West Virginia into Tennessee and, within its Kentucky range, forms the western border of the Cumberland Mountains. Formed from the Pine Mountain thrust fault, its strata dip southeastward forming a dip slope ranging in altitude from approximately 488 to 975 m over a distance of 1.6 to 2.4 km. Small southerly flowing streams that drain into the Poor Fork of the Cumberland River occur at about 0.8 km intervals along the extent of the dip slope (Braun 1935). The eroded edges of the strata make up a steep scarp slope on the northeast aspect of Pine Mountain that is characterized by decreases in altitude of 305 m over a distance of 402 m to 603 m (Braun 1935).

THE STUDY SITE

Hi Lewis Pine Barrens State Nature Preserve is a 68-ha forested tract on the south slope of Pine Mountain, Harlan County, Kentucky. It lies approximately 8 km southwest of the vicinity where Braun (1935) made the majority of her pre-blight observations. Elevation is 488 m at the base of the slope, and increases along a 1.6 km gradient to 792 m at the crest of the mountain. The most widely distributed soil in the transect is an Alticrest-Totz-Helechawa complex described by Childress (1992) as acidic, highly permeable and low in natural fertility. A Helechawa-Varilla-Jefferson complex, a slightly deeper, acidic soil, is also present; however, it is found only in the ravine formed by Ashhopper Branch on the east boundary of the preserve. Underlying bedrock consists of Pennsylvanian sandstones, siltstones and shales (Childress 1992). On average, Harlan County receives 127 cm of precipitation annually, with 50% of this falling in April through September. Mean annual temperature is 12.7 °C (Childress 1992). The growing season is from April through September, with first and last freeze dates occurring in October and April, respectively (Childress 1992).

METHODS

Post-blight Forest Types and Structure

Following observation of topographic maps and aerial photographs and reconnaissance of the area, nine temporary transects (designated 1 through 9) and five permanent (designated P1 through P5) 0.04 ha plots were established. In June and July 1997, data were collected from each of the permanent plots and from 0.04 ha circular sampling plots placed at 30 m intervals along each transect. During data collection, coordinates for each plot were recorded on a Trimble® GPS. Within each 0.04 ha plot, woody stems with a dbh $\geq$ 10.16 cm (canopy) were measured and recorded by species. In a 0.02 ha circular plot nested in the center of each 0.04 ha plot, woody stems with a dbh of 2.54–10.15 cm (subcanopy) were measured and recorded by species, with the

exception of oaks, pines, and hickories, which were identified by genus. These data collected for the canopy and subcanopy strata were used to calculate percent composition, density, relative density, basal area, relative basal area, frequency, and relative frequency. Summation of the relative values gave an importance value (IV) with a maximum of 300 (Curtis and McIntosh 1950; Barbour et al. 1987). Woody groundcover (tree species; dbh < 2.54 cm) within a 0.0025 ha circular plot nested in the center of each 0.04 ha plot were counted and recorded by genus. The data were used to obtain density, relative density, frequency, relative frequency, and importance values (maximum 200) for the woody groundcover stratum.

Community coefficients (CC) for pairwise comparison of all transects and permanent plots were calculated using Horn's index (Horn 1966), with subsequent cluster analysis of CCs to identify distinct forest types. A threshold CC of 0.50 was used to delineate forest types (Barbour et al. 1987). The Shannon-Weiner Index of Species Diversity was calculated for each post-blight forest type for comparison with other forests in the Mixed Mesophytic Region.

Pre- and Post-blight Comparison

A proportional similarity index was used for comparison of post-blight forest types with the 16 pre-blight forest types described by Braun (1935). The author recognizes that Dr. Braun did not give canopy size class diameter. However, she documented percent composition in several different stands and augmented this information with descriptions of elevation, slope, aspect, and edaphic factors. Collectively, this information permits reasonable comparison of pre- and post-blight forest types.

RESULTS

Post-blight Forest Types and Structure

A total of sixteen canopy (dbh ≥ 10.16 cm) species were identified. Cluster analysis of species data collected from transects and permanent plots yielded four distinct forest types: *Liriodendron-Acer, Quercus-Tsuga,* Mixed *Quercus,* and *Pinus-Quercus.* Canopy species diversity was greatest in the *Quercus-Tsuga* and Mixed *Quercus* forest types and least in the *Liriodendron-Acer* and *Pinus-Quercus* forest types (Table 1).

Table 1. Proportional similarity comparison of pre- and post-chestnut blight forest types, and species diversity indices for post-blight forest types of Hi Lewis Pine Barrens State Nature Preserve on Pine Mountain, Harlan County, Kentucky.

1935[a]	1997	Similarity (%)	H'[b]
Chestnut-Tulip	Tuliptree-Red Maple	55.5	1.35
Hemlock	Oak-Hemlock	49.5	2.61
Chestnut Oak-Chestnut-Tulip	Mixed Oak	45.7	3.12
Chestnut Oak-Pine	Pine-Oak	72.9	1.93

[a] Braun, 1935.
[b] 1997.

A narrow, wet-mesic longitudinal trough (Transect 1; N_{plot} = 2; altitude, 768 m) near the crest of the mountain supported a *Liriodendron* dominant canopy (IV = 187.6) with *Acer rubrum* L. (IV = 64.2) second in importance (Table 2). *Liriodendron tulipifera* L. (IV = 50.0) was codominant with *Sassafras albidum* (Nutt.) Nees (IV = 47.3) in the subcanopy (Figure 1A). *Sassafras albidum, A. rubrum,* and *Carya* spp. contributed equally to the woody groundcover stratum (Figure 1A).

In the dip slope ravine (Transect 9; N_{plot} = 4; elevation, 570–573 m) on the east boundary of the preserve, *Quercus alba* L. (IV = 66.0), *Q. rubra* L. (IV = 55.5), and *Tsuga canadensis* (L.) Carr. (IV = 45.5) were codominants in the *Quercus-Tsuga* forest type (Table 2). *Acer rubrum* and *L. tulipifera* also contributed heavily to the canopy with importance values of 45.0 and 44.6, respectively (Table 2). *Acer rubrum* (IV = 45.0) was the most important species in the subcanopy, followed by *Oxydendrum arboreum* (L.) DC. (IV = 39.2), and *S. albidum* (IV = 37.2; Figure 1B). *Acer rubrum* was the dominant woody groundcover species with an importance value of 64.3. Species of lesser importance in this stratum were *S. albidum* (IV = 30.8), *T. canadensis* (IV = 28.7), and *Quercus* spp. (IV = 27.1; Figure 1B).

A mixed *Quercus* forest type (Transects 5, 6; N_{plot} = 5; elevation, 658–768 m) was found on mesic, ESE aspects of the dip slope. *Quercus velutina* Lam. (IV = 71.4) and *Q. montana* L. (IV = 44.8) were codominant canopy species (Table 2). *Quercus* spp. (IV = 25.9) were less important in the subcanopy, where the

Table 2. Canopy (dbh ≥ 10.16 cm) composition and structure for four post-blight forest types of Hi Lewis Pine Barrens State Nature Preserve on Pine Mountain, Harlan County, Kentucky.

	Avg. no. (stems/ha)	Basal area (m^2/ha)	IV (300)
Liriodendron-Acer			
Liriodendron tulipifera	200	19.6	187.6
Acer rubrum	62	2.2	64.2
Morus rubra	25	0.4	26.6
Magnolia fraseri	13	0.1	21.5
Quercus-Tsuga			
Quercus alba	94	0.8	66.0
Quercus rubra	50	0.7	55.5
Tsuga canadensis	56	0.6	45.5
Acer rubrum	38	0.6	45.0
Liriodendron tulipifera	50	0.5	44.6
Quercus montana	50	0.2	34.6
Quercus velutina	6	0.1	8.6
Mixed *Quercus*			
Quercus velutina	35	1.1	71.4
Quercus montana	25	0.7	44.8
Nyssa sylvatica	25	0.4	37.4
Quercus alba	20	0.5	33.9
Liriodendron tulipifera	20	0.5	32.6
Quercus coccinea	20	0.4	31.3
Cornus florida	15	0.1	15.0
Sassafras albidum	10	0.1	12.6
Quercus rubra	5	0.2	12.6
Carya glabra	5	<0.1	8.4
Pinus-Quercus			
Pinus echinata	140	4.3	123.6
Pinus rigida	72	2.9	83.3
Quercus montana	13	0.7	32.0
Quercus coccinea	21	0.7	29.2
Quercus velutina	6	0.3	11.3
Quercus rubra	3	0.3	6.7
Carya glabra	6	0.1	6.6
Quercus alba	3	<0.1	4.3
Robinia pseudoacacia	1	<0.1	3.3

dominant species was *A. rubrum* (IV = 62.0), followed by *S. albidum* (IV = 53.2) and *Castanea dentata* (IV = 43.5). However, *Quercus* spp. (IV = 61.0) were dominant in the woody groundcover, followed by *S. albidum* (IV = 38.7) and *A. rubrum* (IV = 19.7; Figure 1C).

The most extensive forest type at the study site was *Pinus-Quercus* (Transects 2, 3, 4, 7, 8, P 1–5); N_{plot} = 17; elevation, 488–792 m), which was found on dry ridges and areas of the dip slope with a SSE aspect. *Pinus echinata* Mill. and *P. rigida* Mill. were codominant canopy species with importance values of 123.6 and 83.3, respectively (Table 2). Although individual importance values for each *Quercus* spp. appeared minimal relative to those of *Pinus* spp., importance values for five *Quercus* spp. totaled 83.5; therefore, the genus was considered codominant with *Pinus* (Table 2). *Quercus* spp. (IV = 62.5), *A. rubrum* (IV = 61.5), and *Pinus* spp. (IV = 50.8) were codominants in the subcanopy (Figure 1D). *Quercus* spp. (IV = 62.5) were the dominant woody groundcover species, followed in importance by *S. albidum* (IV = 41.4), and *Pinus* spp. (IV = 21.6; Figure 1D).

Pre- and Post-blight Comparison

Without *Castanea dentata* in the canopy, the four forest types delineated in this study showed moderate to high proportional similarity with four described by Braun (1935) at the onset of chestnut blight in southeastern Kentucky (Table 1). Braun's (1935) Chestnut-Tulip forest type, found in longitudinal troughs at the crest of Pine Mountain, had the greatest proportional similarity (55.5%) to the present-day Tuliptree-Red Maple (*Liriodendron-Acer*) type (Table 1). Pre-blight, *C. dentata* contributed up to one-third of the canopy in the Chestnut-Tulip forest type (Braun, 1935). In the present study, *C. dentata* was found only in the subcanopy of the *Liriodendron-Acer* forest type, where it ranked eighth in importance (IV = 20.9; Figure 1A).

The current Oak-Hemlock (*Quercus-Tsuga*) forest type showed greatest proportional similarity to Braun's (1935) Hemlock forest type (Table 1). *Castanea dentata* contributed only 4% to the pre-blight Hemlock forest (Braun 1935). In this study, it was not found in the *Quercus-Tsuga* canopy of the dip slope ravine (Table 2), ranked tenth in importance in the subcanopy, and was not found in the woody groundcover stratum (Figure 1B).

The proportional similarity index was greatest between the current Mixed Oak (*Quercus*) forest type and Braun's (1935) Chestnut Oak-Chestnut-Tulip forest type (Table 1). Prior to chestnut blight, *C. dentata* was a codominant contributing up to one-third to the Chestnut Oak-Chestnut-Tulip canopy composition (Braun, 1935). In the current Mixed Oak forest type, *C. dentata* is absent from the canopy (Table 2), a codominant (IV = 43.5) in the subcanopy, and ranked fifth (IV = 10.1) in importance among woody groundcover species (Figure 1C).

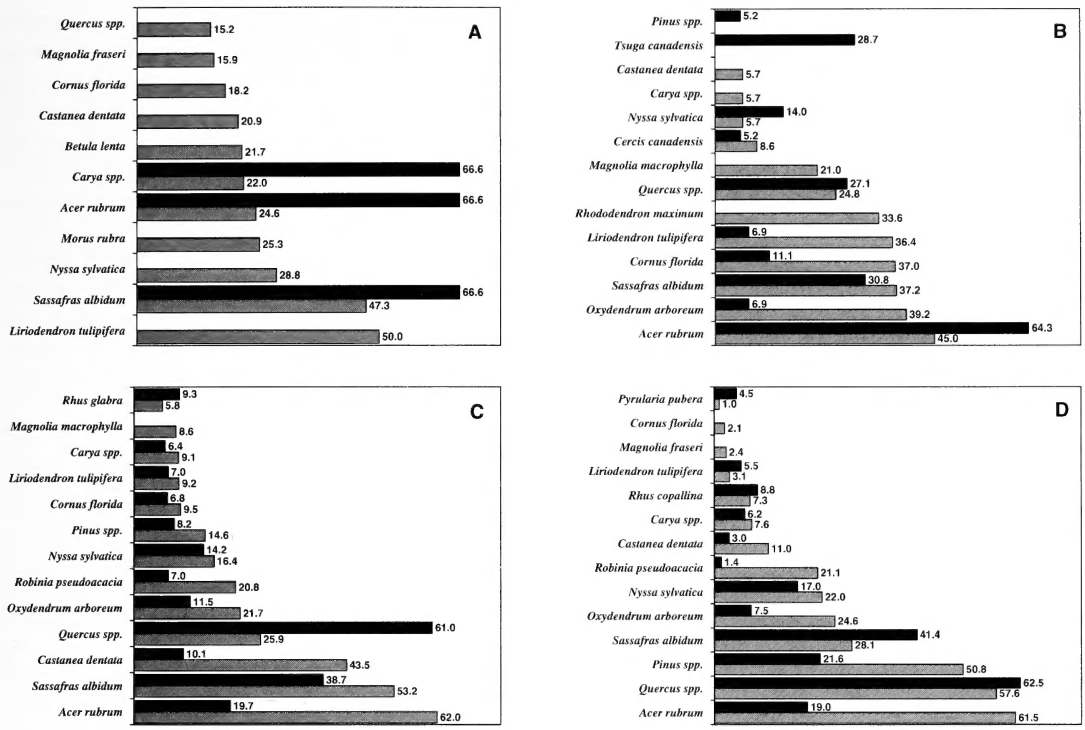

Figure 1. Importance values for subcanopy (dbh 2.54–10.15 cm; grey bars) and woody groundcover (<2.54 cm; black bars) strata in A) *Liriodendron-Acer,* B) *Quercus-Tsuga,* C) Mixed *Quercus,* and D) *Pinus-Quercus* post-chestnut blight forest types of Hi Lewis Pine Barrens State Nature Preserve on Pine Mountain, Harlan County, Kentucky.

Composition of the Pine-Oak (*Pinus-Quercus*) forest type identified in this study showed little difference (Proportional Similarity Index = 72.9%) to the Chestnut Oak-Pine forest described by Braun (1935; Table 1). Based on percent composition, *C. dentata* made up approximately 11% of the pre-blight Chestnut Oak-Pine canopy (Braun, 1935); however, *C. dentata* was not present in the *Pinus-Quercus* canopy and ranked eighth (IV = 11.0) and eleventh (IV = 3.0) in importance values in the subcanopy and woody groundcover layers, respectively (Figure 1D).

DISCUSSION

Keever (1953), Stephens and Waggoner (1980), and Russell (1987) have shown that *Castanea dentata* remains an important component of plant communities throughout its natural range. Further, its persistence in many post-blight forests results from root-crown sprouts from pre-blight root systems (Russell 1980; Paillet 1982, 1984; Schwadron 1995; Schibig et al. 2003). The present study identified four distinct forest types: *Liriodendron-Acer, Quercus-Tsuga,* Mixed *Quercus,* and *Pinus-Quercus.* Although *C. dentata* is no longer part of the canopy, it is found in the woody groundcover stratum in two of the four forest types and in the subcanopy in all four forest types. Further, it is likely that *C. dentata* will remain a part of these forest communities, persisting from root crown sprouts.

In the post-blight years, forest succession on this section of the dip slope of Pine Mountain has followed a pattern similar to that reported for forests in Braun's (1950) Western Mesophytic (Schibig et al. 2003; Myers et al. 2004) and Oak-Chestnut Region (Keever 1953; Woods and Shanks 1959; Good 1968; Christensen 1977; Karban 1978; Arends 1981; Johnson and Ware 1982; Stephenson 1986; Stephenson et al. 1991; Hannah 1993; Parker et al. 1993). In general, gaps created by dying chestnut have been filled by codominant pre-blight canopy species, with minor invasion by subcanopy species. However, the extent of change in canopy composition in the four for-

est types appears to be consistent with the extent to which chestnut was lost. For example, the forest type least changed by chestnut decline is the present-day *Pinus-Quercus* forest type that is very similar to Braun's (1935) Chestnut Oak-Pine forest type. Braun (1935) did not consider *C. dentata* to be an important component of the Chestnut Oak-Pine canopy. *Pinus echinata* and *P. rigida* were codominants, making up approximately 50% of canopy composition, and she described *Quercus montana* (~21% canopy composition) as important (Braun 1935). Based on importance values and percent composition from this study, these three species remain codominant.

Similarly, *C. dentata* was not important in Braun's (1935) Hemlock forest type found in the dip slope ravines of Pine Mountain. Currently, *Tsuga canadensis* remains important in the *Quercus-Tsuga* forest type of the dip slope ravine, although the species ranks slightly subordinate to *Q. alba* and *Q. rubra.* The results suggest that in the dip slope ravine, chestnut decline may have allowed for subordinate oak species to enter the canopy. This pattern of chestnut replacement was documented for post-blight forests in Braun's Oak-Chestnut region. Karban (1978), Johnson and Ware (1982), and Stephenson (1986) described chestnut replacement by a single oak species, such as *Q. rubra* or *Q. montana.* However, *C. dentata* was considered a codominant in those pre-blight forests; whereby it was of little importance to the pre-blight Hemlock forest type (Braun 1935).

Chestnut replacement was most apparent on ESE aspects of the dip slope where the Mixed-*Quercus* forest type is only remnant of Braun's (1935) Chestnut Oak-Chestnut-Tulip forest type descriptions. The importance of *C. dentata* to the pre-blight forest is still evidenced by its codominance in the subcanopy, as well as by being the sixth most important tree species in the woody groundcover. Within this forest type, *C. dentata* has been replaced by *Q. velutina,* and to a lesser degree, by *Q. montana.* Replacement of chestnut by codominant oaks also has been described for forests in the Great Smoky Mountains (Woods and Shanks 1959; Arends 1981; Golden 1981; Parker et al. 1993). Further, trees that Braun (1935) did not consider canopy species in the pre-blight Chestnut Oak-Chestnut-Tulip forest are found in the Mixed *Quercus* canopy, suggesting that some canopy gaps may have allowed for invasion of non-canopy species such as *Nyssa sylvatica* Marsh., *Cornus florida* L., and *Sassafras albidum.* This pattern of chestnut replacement has also occurred in mesic forests in West Virginia and North Carolina (Stephenson 1986; Hannah 1993) and in former chestnut occupied forests of the northern Highland Rim of Kentucky and Tennessee (Schibig et al. 2003). In these forests, species formerly considered non-canopy trees, such as *Acer rubrum, C. florida, Betula lenta* L., *Oxydendrum arboreum, N. sylvatica, Prunus serotina* Ehrend., and *Robinia pseudoacacia* L. entered the canopy following chestnut death (Stephenson 1986; Hannah 1993; Schibig et al. 2003).

In the longitudinal trough at the crest of Pine Mountain, the codominant *C. dentata* of Braun's Chestnut-Tulip forest type has been replaced primarily by *A. rubrum.* Although *Morus rubra* L. contributed to the present-day *Liriodendron-Acer* forest type, it is more often found on the northwest slope of Pine Mountain. On the other hand, presence of *Magnolia fraseri* Walt. in the subcanopy is typical of mesic areas in the mixed mesophytic forests of the Cumberland Plateau (Braun 1942).

Chestnut death appears to have precipitated relatively minor changes in forest composition on Pine Mountain; however, the influence of other factors such as aspect, physiography, and anthropogenic disturbance on post-blight succession should be taken into consideration in assessing current status and predicting future changes in these forest types. In the Appalachian Plateau Region the microclimate of a site is strongly influenced by its slope and aspect (Franzmeier et al. 1969; Hutchins et al. 1976). In turn, soil moisture (Whittaker 1956; Cooper and Hardin 1970; Day and Monk 1974; McEvoy et al. 1980) or a combination of soil moisture and fertility (Muller 1982) are important factors in determining patterns of species distribution and vegetation structure in southern Appalachian forests. Braun (1935) described a mosaic of forest communities on Pine Mountain resulting from variability in edaphic and topographic conditions over relatively short distances. Similarly, forest types identified in the present study changed with shifts in aspect, and change from one forest

type to another was often abrupt. For example, sampling plots with a SSE aspect and those on ridges were xeric and supported a *Pinus-Quercus* canopy; whereby, *Lirodendron-Acer* was found in the most mesic site at the crest of Pine Mountain. With change in aspect from SSE to ESE, *Pinus* spp. dropped out of the canopy and *Quercus* spp. were codominant in these more mesic plots.

Subcanopy composition appeared less influenced by changes in aspect and slope than the canopy. Although *Pinus* spp. were restricted to a single aspect (SSE), *Quercus* spp., *A. rubrum,* and *C. dentata* were found in all forest types. Species described as fire-sensitive, such as *N. sylvatica, O. arboreum,* and *S. albidum* (Martin 1989; Delcourt and Delcourt 1997) were part of the subcanopy and woody groundcover in at least three of four forest types. Frequency and importance of these latter three species may be attributed, in part, to frequent fire disturbance.

Diversity indices (H′) for the Mixed *Quercus* and *Quercus-Tsuga* forest types fall within the range of values (2.02–3.4) reported by Monk (1967) for forest types in the Mixed Mesophytic Forest Region. By contrast, forest types found on the most xeric (*Pinus-Quercus*) and most mesic (*Liriodendron-Acer*) areas of the dip slope had diversity indices below those recorded for forest types of the Mixed Mesophytic Forest Region (Monk 1967; Martin 1992; Clinton et al. 1993). Given that the *Liriodendron-Acer* forest type reflects chestnut replacement by a single species (*A. rubrum*), and little change occurred in the shift from Braun's Oak-Pine forest to the current Pine-Oak forest, these depressed diversity indices may well be the result of physiography, and not chestnut death.

Except for the absence of *C. dentata* in the canopy, forest composition on this section of Pine Mountain has remained relatively unchanged in the past 70 years. However, chestnut blight did create changes in the relative importance of pre-blight tree species, resulting in varying degrees of transition to post-blight forest types. Given the influence of aspect and physiography on species distribution and forest structure of southern Appalachian forests, current composition and relative importance of post-blight forest species would be expected to show minimal change in future years. However, the importance of fire-sensitive, as well as other non-canopy species, in the lower forest strata suggests that continued fire disturbance (i.e., arson) in conjunction with microhabitat may allow for subordinate tree species to enter the canopy and precipitate further change in the post-blight forest types.

ACKNOWLEDGMENTS

The author would like to thank Donald Barnes, Robert Hawkins, and Kyle Napier for their field assistance, the Kentucky State Nature Preserves Commission for underwriting the cost of this research, and Drs. Edward W. Chester, Steve Meadows, and Jeffrey Walck for critical reviews of an earlier draft of this manuscript.

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J. Ky. Acad. Sci. 67(2):81–86. 2006.

Thirty Years of Recovery in a Tornado-damaged Forest in Northern Kentucky

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ABSTRACT

This 30–year study reports on the changes in the structure and composition of an old-growth forest damaged by a tornadic windstorm in 1974. Tree density, basal area and species composition were measured at Dinsmore Woods State Nature Preserve, Boone County, Kentucky in 1973, in 1974 at the time of the tornado, and in 1985, 1994 and 2004 to document and assess community change in response to this disturbance. There was an initial decline in both density (N/ha) and basal area (m^2/ha), followed by gradual increases, and by 2004 the forest community composition was similar to that of the pre-tornado community. *Acer saccharum* had experienced increases in both the overstory and understory strata while *Quercus* spp. and *Fraxinus americana* were the major overstory subdominants. These two species showed a decline in density in the understory.

KEY WORDS: forest recovery, windstorms, Dinsmore Woods Sate Nature Preserve, Boone County

INTRODUCTION

Windstorms, such as tornados, often have profound effects on forests, and these storms may alter community structure and composition (Wright 1974; Dunn et al. 1983; Glitzenstein and Harcombe 1988; Foster 1989; Peterson and Pickett 1995; Everham and Brokaw 1996). Windstorms often create gaps in the forest canopy that may alter the successional sequence by allowing propagules of earlier successional species to enter into the community (Runkle 1982, 1984) or by enhancing the development of stems previously suppressed under a closed canopy (Canham 1985; Canham and Marks 1985)

In spring 1973 a preliminary study of the forest community at Dinsmore Woods, a 43.3 ha forest in Boone County, Kentucky (Figure 1), was completed by the senior authors (Held and Bryant). On 3 April 1974, a tornado damaged a portion of this forest. A comprehensive survey of this recently disturbed forest community was completed in June and July 1974 (Held 1975; Held and Winstead 1976). This survey allowed an assessment of the forest community structure and composition using both tornado-damaged and undamaged portions of the forest. However, delayed effects of the storm on the forest community, i.e., damaged trees that would survive and apparently undamaged trees that would fall later, were not considered.

At approximately 10-year intervals following the 1974 survey (1985 and 1994), the forest was resurveyed with a focus on changes in community composition (Held and Bryant 1989; Held et al. 1998). Again, in 2004 the forest was surveyed in a continuing assessment of forest recovery process.

Our objective was to continue examination of long-term changes in community composition of a wind-damaged forest located at the southern terminus of the glacial advance in northern Kentucky. Few studies have examined long-term recovery patterns of forest communities damaged by catastrophic windstorms (Everham and Brokaw 1996). Also, the

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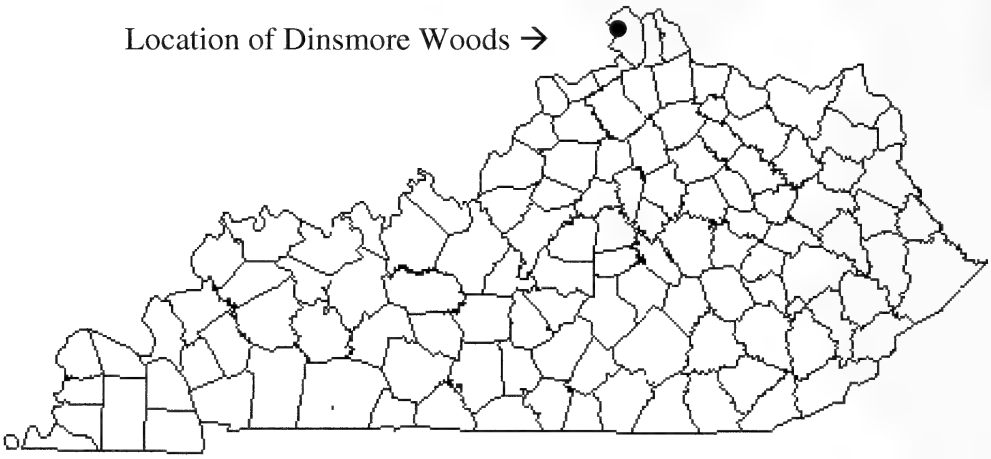

Figure 1. Boone County, KY and location of Dinsmore Woods.

extent of the damage at Dinsmore Woods was patchy, which is unlike the usual situation of massive blowdowns that characterize most catastrophic windstorms in forests (Peterson and Pickett 1995).

STUDY SITE

Dinsmore Woods is located in western Boone County, Kentucky (39.001° N, 84.815° W) approximately 8 km west of Burlington and 3.2 km east of the Ohio River (Figure 1). The forest is located on moderate to steep slopes approximately 220–250 m above the Ohio River floodplain. The bedrock of Boone County is primarily Ordovician limestone and shale (McFarlan 1943) and is covered by Pleistocene glacial till deposits (Braun 1936) and outwash of Illinoian and Kansan age till (Ray 1974). The Woods is presently owned by The Nature Conservancy and is a Kentucky State Nature Preserve.

The 1973 and 1974 sampling data indicated the site to be a mesic hardwood forest dominated by *Acer saccharum* with *Fraxinus americana* and *Quercus* spp. as subdominant species (Held 1975). The understory consisted of much the same species as the canopy (Held 1975). Prior to the spring of 1974 there were no historical accounts or indications of any significant modification of the site since the land was obtained by private ownership in the late 1830's (M. Breasted, pers. comm. 1974).

MATERIALS AND METHODS

For each survey, a nested circular plot sample method was used (Ohmann 1973). Species and diameter at breast height or DBH (measured at 1.37 m above ground) of trees ≥10 cm DBH were recorded in 1973 in 14 plots 0.04 ha in size. In 1974, following the tornado, 23 plots of 0.125 ha were sampled. Fifteen 0.04 ha plots were surveyed in 1985, 1994, and 2004. The plots sampled in 1985, 1994, and 2004 were located within or as close as possible to the 1974 plots and were within 20 m of the original transect lines used for the placement of the 1974 plots.

Seedlings and saplings were recorded in plots nested within the tree plots for the four sampling dates (seedling and sapling data were not collected in 1973). In 1974, the seedling and sapling plots were 0.031 ha and 0.062 ha, respectively. For the three subsequent surveys, plot size for seedlings was 0.004 ha and 0.01 ha for saplings. During data collection seedlings and saplings were placed into five size classes (Held et al. 1998). However, for analysis, Class I, II, and III stems were grouped into one size class (seedlings–young saplings), with Class IV and V individuals in another group (saplings). Nomenclature followed Fernald (1950).

Species composition and relative density by size class (tree, saplings, and seedlings) were determined. Relative basal area was calculated for the tree stratum, summed with relative

Table 1. Forest community characteristics for Dinsmore Woods in 1973 (pre-tornado), 1974 (post-tornado), 1985, 1994, and 2004 surveys.

	1973	1974	1985	1994	2004
Number of tree species	16	21	15	13	17
Density (N/ha)	334	320	242	261	285
Basal area (m^2/ha)	25	28	19	22	30

density, and divided by two to give an importance percentage for each species (IP = (RD + RBA)/2). A similarity coefficient (Bray and Curtis 1957) was used to compare the stand at different sample dates. The coefficient was defined as: $C = (2W/a + b) \times 100$, where "a" was the sum of the importance percentages of all tree species at one time period, "b" was the sum of the importance percentages for the second time period and "W" was the sum of the lower importance percentages for each species recorded at both time periods.

RESULTS

Overall, the density of trees at Dinsmore Woods decreased by 78 trees/ha following the 1974 tornado to 242 trees/ha in 1985; that number increased to 261 trees/ha in 1994 and to 285 trees/ha in 2004 approaching the pre-tornado value. A similar trend was observed in total basal area (m^2/ha) with a drop to 19 m^2/ha in 1985, followed by an increase to 22 m^2/ha in 1994 and had, by 2004 (30 m^2/ha), exceeded the pre-tornado basal area value (Table 1). Diameter size-class distributions followed similar inverse J-shapes for the 1974 and 2004 sampling periods (Figure 2). Between 1974 and 2004 the decline in density of smaller diameter trees (10–20 cm size classes) was noticeable.

The similarity coefficient (C) between the 1973 survey and 1974 surveys was 86%, indicating high species similarity and density distribution between the pre-tornado and tornado surveys. That similarity value fit the coef-

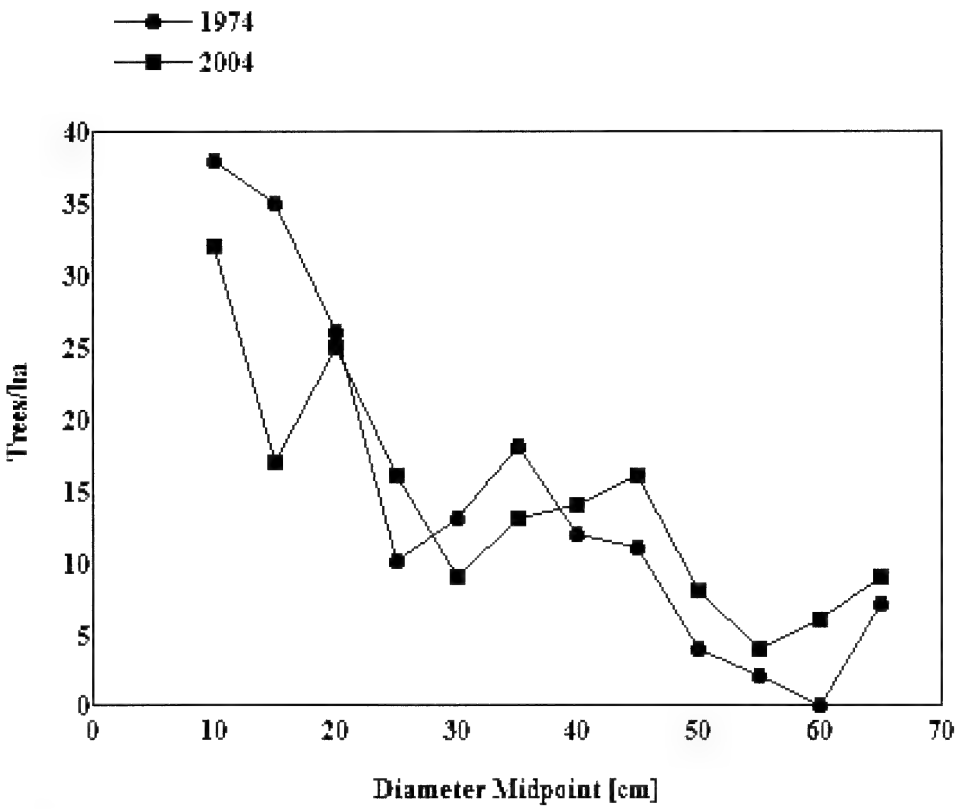

Figure 2. Diameter distribution of trees by diameter class at Dinsmore Woods in 1974 and 2004.

Table 2. Dinsmore Woods community coefficient matrix (based on relative density) across the four sampling periods.

	1973	1974	1985	1994	2004
1973	—				
1974	86.0	—			
1985	*	68.6	—		
1994	*	68.0	78.4	—	
2004	*	68.1	80.9	75.0	—

* Comparison not possible.

ficient (C ≥ 80%) proposed by Bray and Curtis (1957) for samples of same forest. However, the similarity between 1974 and 2004 was only 68.1% indicating either compositional or structural changes or both in the forest community following the tornado. After that initial drop, similarity increased between the first and subsequent sampling periods (Table 2) suggesting that the forest had remained fairly similar in terms of composition and density.

Acer saccharum density increased from 114 trees/ha in 1974 to 128 trees/ha in 1985 to 158 trees/ha in 1994, after which it leveled off to 157 trees/ha in 2004 (Table 3). The oaks (*Quercus* spp.) have remained fairly stable over the 30 years following the disturbance. After 30 years of decline in density, *Fraxinus americana* was now nearing its 1974 density. However, the remaining tree species declined in density from 59 trees/ha in 1974 to 26 trees/ha in 2004 (Table 3).

Acer saccharum has remained the dominant understory species (Table 4). Among the subdominants of the canopy, only *Celtis occidentalis* showed an increase in 2004 in the smaller size class. In the larger size class, no subdominant species showed an increase in 2004, and most were absent from this stratum. Of those small tree/shrub species usually not recorded in the tree layer, *Asimina triloba* and *Lindera benzoin* showed a large increase in total understory stems from 1974 to 2004 (Table 4).

DISCUSSION

Windstorms affect forest communities in various ways. Changes in the forest community initiated by windstorms are dependent on three interrelated processes: 1) the initial change to the forest community caused by intense winds; 2) subsequent change due to the

Table 3. Density (N/ha), relative density (RD), relative basal area (RBA), and importance percentage (IP) for the tree stratum (IP ≥ 5.0) at Dinsmore Woods in 1974, 1985, 1994, and 2004.

Species	N (1974)	N (1985)	N (1994)	N (2004)	RD (1974)	RD (1985)	RD (1994)	RD (2004)	RBA (1974)	RBA (1985)	RBA (1994)	RBA (2004)	IP (1974)	IP (1985)	IP (1994)	IP (2004)
Acer saccharum	114	128	158	157	35.6	52.9	60.5	55.1	34.4	35.5	46.2	36.9	35.0	44.2	53.4	46.0
Fraxinus americana	46	23	20	40	14.4	9.5	7.7	14.0	15.7	24.0	8.8	23.3	15.1	16.8	8.3	18.7
Celtis occidentalis	35	7	12	8	10.9	2.9	4.6	2.8	9.3	2.7	3.2	1.7	10.1	2.8	3.9	2.2
Ulmus rubra	32	25	22	14	10.0	10.3	8.4	4.9	4.7	6.8	3.7	4.3	7.4	8.6	6.0	4.6
Quercus alba	12	2	25	2	3.8	0.8	9.6	0.7	7.3	1.5	21.1	1.1	5.5	1.2	15.3	0.9
Carya cordiformis	9	5	5	13	2.8	2.1	1.9	4.6	4.7	5.3	1.6	5.1	3.8	3.7	1.8	4.8
Quercus rubra	3	11	2	4	0.9	4.5	0.8	1.4	4.3	13.0	7.7	3.7	2.6	8.8	4.2	2.5
Juglans nigra	7	3	0	11	2.2	1.2	0.0	3.9	1.4	2.5	0.0	7.0	1.8	1.8	0.0	5.5
Quercus muhlenbergii	3	10	0	10	0.9	4.1	0.0	3.5	1.9	6.2	0.0	6.2	1.4	5.1	0.0	4.8
Other species*	59	28	17	26	18.5	11.7	6.5	9.1	16.3	2.5	7.7	10.7	17.3	7.0	7.1	10.0
Total	320	242	261	285	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

* *Gleditsia triacanthos, Ostrya virginiana, Fagus grandifolia, Cercis canadensis, Acer negundo, Tilia americana, Robinia pseudoacacia, Maclura pomifera, Prunus serotina, Quercus prinus, Cornus florida, Aesculus glabra, Asimina triloba, Liriodendron tulipifera, Ulmus americana, Fraxinus quadrangulata, Gymnocladus dioicus.*

Table 4. Comparison of understory (seedlings and saplings) absolute density (stems/ha) in 1974, 1985, 1994, and 2004.

Species	Classes I–III				Classes IV–V			
	1974	1985	1994	2004	1974	1985	1994	2004
Acer saccharum	2333	2567	153	2043	448	107	13	113
Ulmus rubra	1458	429	55	396	385	53	2	0
Fraxinus americana	833	286	5	133	125	0	0	0
Lindera benzoin	368	949	41	2324	59	0	0	0
Prunus serotina	319	56	2	33	56	33	0	0
Asimina triloba	264	1768	29	1530	219	120	0	0
Carya spp.	257	208	10	67	45	0	0	0
Celtis occidentalis	250	754	2	396	97	14	0	7
Quercus spp.	181	13	0	53	38	0	0	0
Gleditisia triacanthos	125	0	3	0	0	0	0	0
Sassafras albidum	83	0	0	0	3	7	0	0
Ostrya virginiana	42	0	0	0	28	7	0	0
Cornus florida	28	13	0	0	24	0	0	0
Tilia americana	21	208	0	0	7	39	0	7
Fagus grandifolia	14	13	0	20	3	0	0	0
Cercis canadensis	14	56	0	0	10	7	0	0
Juglans nigra	7	0	0	0	0	0	0	0
Morus rubra	7	13	0	0	17	0	0	7
Robinia pseudoacacia	0	0	2	0	3	0	0	0
Liriodendron tulipifera	0	0	0	0	3	0	0	0
Eunymous americana	0	0	0	0	3	0	0	0
Aesculus glabra	0	42	0	13	0	0	0	0
Symphoricarpos orbiculatus	0	42	0	0	0	0	0	0
Fraxinus quadrangulata	0	0	0	83	0	0	0	0
Total (stems/ha)	6604	7417	302	7091	1573	387	15	134

Class I = seedlings from 15 cm to 1.4 m in height; Class II = saplings over 1.4 m in height with diameters less than 1.27 cm; Class III = saplings over 1.4 m in height with diameters from 1.28 to 3.81 cm; Class IV = saplings over 1.4 m in height with diameters from 3.82 to 6.35 cm; Class V = saplings over 1.4 m in height with diameters from 6.36 to 9.99 cm.

death of trees originally damaged but not killed by the storm; and 3) the mosaic pattern of regrowth that results from the interaction of the first two events. The initial change is also dependent upon variations in topography, soils, storm intensity and duration, and the physical tolerances of trees to intense winds. Further, the vegetation response to large blowdowns caused by intense windstorms is quite different from single gaps, as the former open much of the canopy and disturb soils, favoring early successional species (Dunn et al. 1983). Large blowdown areas were not created at Dinsmore Woods; instead, smaller blowdown areas or gap openings were formed by uprooted trees and trees whose canopy area was damaged and reduced.

While no dominant tree species were lost from the forest community during the 30 years following the tornado, changes in either density or basal area or both in most species were readily apparent. *Acer saccharum* maintained its dominance in both the canopy and understory. The continued and increasing importance of this species may be accounted for by two factors: 1) release and growth of suppressed seedlings, saplings and small trees into newly created gaps (Canham 1985) and 2) the large mast of winged fruits may give this species a competitive advantage over other genera (i.e., *Quercus* spp.) in exploiting canopy openings. Also, as canopy gaps close, shade-tolerant *Acer saccharum* has an advantage over other less shade tolerant gap species (Dahir and Lorimer 1996). Dahir and Lorimer (1996) reported that after 40 years of recovery from a windstorm to a northern hardwood forest in Michigan, there was little difference in the level of dominance of maple between the 40-year old recovery forest and an old-growth uneven-aged community in the same region. A similar pattern for *Acer saccharum* seems to be emerging at Dinsmore Woods (Table 3).

Tree seedling establishment also shows this pattern of dominance by *Acer saccharum.* Immediately following the tornado there was a burst of growth in the understory with many species contributing to this (Table 4). By 2004

most of the seedlings and saplings were represented by *Acer saccharum.* While *Asimina triloba* and *Lindera benzoin* usually do not enter into the tree stratum, these species may affect further changes in the forest the forest composition by way of competition with tree seedlings, thus possibly altering the understory composition.

Braun (1916, 1950) described the forests of this region as Oak–Maple–Ash. In this forest, maple was dominant in 1974 followed by ash and oak as subdominants and those species were dominant 30 years following the tornado. However, there has been a shift toward increased importance of *Acer saccharum.* This species not only has maintained but has expanded its dominance at this site 30 years following the tornado. That dominance also occurs in the understory to the near exclusion of all other tree species. This species seems to have tolerated the disturbance better than most species in the community especially benefiting from release (Abrams and Scott 1989).

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J. Ky. Acad. Sci. 67(2):87–92. 2006.

Index Herbariorum Kentuckiensis IV

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ABSTRACT

A survey conducted in August and September 2006 provides information on present herbarium collections at 12 Kentucky institutions. A total of 338,000 vascular and nonvascular specimens are reported. Of these, 324,700 are vascular specimens, and 13,400 are nonvascular. This number represents a 37% increase in specimens since the last survey in 1995. Updates are provided on current curators, databasing efforts, and other changes during the last decade.

KEY WORDS: Herbaria, curators, databases in Kentucky, vascular plants, nonvascular plants

INTRODUCTION

This paper is the fourth article dealing with the status of herbarium collections in Kentucky. Earlier papers in the series were published by Lassetter (1978), Jones (1987), and Jones et al. (1995). In these articles, the following numbers of specimens were reported, respectively, 119,000 at 12 institutions, 187,000 at 15 institutions, and 246,000 specimens at 13 institutions. The current survey was conducted by sending a survey form to all public and private institutions known to house herbarium collections. Private collections were excluded. The survey was conducted in August and September of 2006. In addition to the standard information requested in earlier surveys, i.e., names, addresses, phone numbers, and fax numbers, numbers of specimens, and special collections and interests, a number of additional items were added for this survey. These included types of databases being used, addresses for herbarium web sites, information on adhesives and insect repellents, use of freezing to prevent infestations, numbers of student workers and departmental funds available, numbers of cabinets and room sizes, and comments on recent or upcoming changes relating to the herbarium.

The results of the survey are provided by city for comparison with previous papers in this series in the listing below, and by institution (for ready reference) in Table 1. The acronyms (in parentheses after the institution) based on Holmgren and Holmgren (1998) are included in the city listing.

Berea

Berea College Herbarium (BEREA). Established in 1961. 20,000 vascular plant specimens. Elmer's Glue-All® Multi-Purpose Glue; Prescription Treatment® Cy-Kick® Crack and Crevice Pressurized Residual® (active ingredient: Cyfluthrin, a pyrethroid insecticide) from Whitmire Micro-Gen Research Laboratories, Inc.; no freezing regime. One student worker per semester; departmental funds average $500–1000/year. Herbarium cabinets number 24, no compactors; in 550 sq. ft. room. Special collections: Berea College Forest (Ralph Thompson, David Taylor); preliminary collections from Bell, Harlan, Laurel, Madison, and Rockcastle Counties (Thompson); floras of surface-mined areas, limestone quarries; Hancock Biological Station of Murray State University; Elk and Bison Prairie, Land Between The Lakes; Rock Creek Research Natural Area; John B. Stephenson Memorial Forest State Nature Preserve, Pine Mountain Settlement School (Thompson); Fort Boonesborough State Park and Hazeldell Sundew Meadow (Richard Abbott). On-going projects: flora of Berea College Forest; floras of Laurel, Madison County, and Rockcastle

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Table 1. Survey data for Kentucky herbaria.

Institution	Herbarium address/web site	Phone number	Fax Number	Curator/email address	Number of specimens: vascular plants (VP), nonvascular plants (NP), databased (DB)
Asbury College	Herbarium, Asbury College, Department of Biology, Wilmore, KY 40390	859-858-3511, ext. 2360	859-858-3921	Ann Witherington ann.witherington@asbury.edu	350 VP, 5 NP, 0 DB
Berea College	Herbarium, Berea College, Department of Biology, CPO Box 2121, Berea, KY 40404	859-985-3378	859-985-3303	Ralph L. Thompson ralph_thompson@berea.edu	20,000 VP, 0 NP, 0 DB
Campbellsville University	Biological Collection, Science Building Rm 101, Campbellsville University, 1 University Drive, Campbellsville, KY 42718	270-789-5043	270-789-5170	Brenda S. Tungate bstungate@campbellsville.edu	1480 VP, 0 NP, 0 DB
Eastern Kentucky University	Herbarium, Eastern Kentucky University, Department of Biological Sciences, 521 Lancaster Ave., Richmond, KY 40475 http://people.eku.edu/jonesron	859-622-6257	859-622-1399	Ronald L. Jones ron.jones@eku.edu	70,000 VP, 500 NP, 27,000 DB
Georgetown College	Herbarium, Georgetown College, Department of Biological Sciences, Georgetown, KY 40324.	502-863-8367	502-868-7744	Timothy Griffith tgriffi0@georgetowncollege.edu	1000 VP, 0 NP, 0 DB
Morehead State University	Herbarium, Morehead State University, Department of Biological and Environmental Sciences, Morehead State University, Morehead, KY 40351	606-783-2587	606-783-5002	Allen C. Risk a.risk@morehead-st.edu	17,318 VP, 9400 NP, 0 DB
Murray State University	Herbarium, Murray State University, Department of Biological Sciences, Murray State University, Murray, KY 42071 www.murraystate.edu/qacd/cos/hbs/herbarium.htm	270-809-4896	270-809-2788	Dayle Sarr dayle.sarr@murraystate.edu	40,500 VP, 2000 NP, 24,447 DB
Murray State University	Hancock Biological Station, Murray State University, Murray, KY 42071 www.murraystate.edu/qacd/cos/hbs/Hancock_Herbarium_Search.cfm	270-474-2272	270-474-0120	Contact David White david.white@murraystate.edu	1200 VP, 0 NP, 1174 DB
Northern Kentucky University	John W. Thieret Herbarium, Department of Biological Sciences, DWH Natural Science Center 204D, Northern Kentucky University, Highland Heights, KY 41099	859-572-1411	859-572-5639	Maggie Whitson whitsonma@nku.edu	35,000+ VP, 0 NP, 100 DB
University of Kentucky	Herbarium, The University of Kentucky, 217b TP Cooper, Dept. of Forestry, Herbarium: Insectory/Conservatory Building, Washington Avenue, University of Kentucky, Lexington, KY 40546 http://www.ca.uky.edu/forestry/herbarium.php	859-257-3094	859-257-7611	Robert Paratley rparatl@uky.edu	57,800 VP, 1500 NP, 0 DB

Table 1. Continued.

Institution	Herbarium address/web site	Phone number	Fax Number	Curator/email address	Number of specimens: vascular plants (VP), nonvascular plants (NP), databased (DB)
University of Kentucky College of Agriculture	Herbarium, College of Agriculture, Department of Plant and Soil Science, 117 Plant Science Building, University of Kentucky, Lexington, KY 40506	859-257-4898	859-257-7874	J. D. Green jdgreen@uky.edu	21,000 VP, 0 NP, 0 DB
University of the Cumberlands	Herbarium, University of the Cumberlands, Department of Biology, 7196 College Station Drive, Williamsburg, KY 40769	606-539-4399	606-539-4319	Todd Yetter tyetter@ucumberlands.edu	3500 VP, 9 NP, 2696 DB
Western Kentucky University	Herbarium, Western Kentucky University, Department of Biology, 1906 College Heights Blvd. #11080, Bowling Green, KY 42101-1080 http://biodiversity.wku.edu/search/plants.asp	270-745-8864	270-745-6856	Lawrence A. Alice lawrence.alice@wku.edu	55,646* VP, 0 NP, 21,644 DB

* Includes specimens to be transferred from the University of Louisville.

Counties (Thompson). Visitation with curator permission. Over 4100 Missouri, Oregon, and Tennessee mounted and labeled collections have been sent to respective state regional herbaria because of space factors.

Curator, Ralph L. Thompson (floristic surveys of specific natural areas of interest).

Bowling Green

Herbarium of Western Kentucky University (WKU). Established in 1967. 25,646 vascular plant specimens. Microsoft Access and Index Kentuckiensis used for databasing. Lineco Inc., Neutral pH Adhesive (99295 53072)®; solid bug strips insect repellent; no freezing regime. No student workers; departmental funds average $200/year. Herbarium cabinets number 30 full-sized and 12 half-sized, no compactors; in 1292 sq. ft. room. Special collections: E. O. Beal, R. Athey, G. Johnson, Max E. Medley. On-going projects: mounting and databasing of Max Medley collection, ~43% complete; acquisition of University of Louisville Davies Herbarium (DHL), which will increase the specimen totals to about 55,000. Contact curator for loan and visitation policies.

Curator, Lawrence A. Alice (*Rubus,* worldwide). **Assistant Curator,** Robert Neidlinger.

Campbellsville

Biological Collection. Established in 1965. 1480 vascular plant specimens. One student worker per semester. On-going projects: Inventory of Clay Hill Memorial Forest. No loan or visitation policies established.

Curator, Brenda S. Tungate (*Trillium,* spring flora, Clay Hill Memorial Forest).

Georgetown

Herbarium of Georgetown College. Established in 1945. 1000 vascular plant specimens. Herbarium cabinets number 6, in a 300 sq. ft. room.

Curator, Timothy Griffith (plant geography, invasive species).

Highland Heights

John W. Thieret Herbarium (KNK). Established 1973. Over 35,000 vascular plant specimens. Elmer's Glue-All® Multi-Purpose Glue; ProZap Pest Guard Strips® insect repellent; minus 9°C for 2–5 days for freezing regime.

One student worker per semester; departmental funds average $500/year. Herbarium cabinets number 50, on compactors, in a 972 sq. ft. room (shared with some zoological and phycological collections, and includes about 25% teaching/work space), with 431 sq. ft. prep room, a 197 sq. ft. work room with library and high quality dissecting scopes, and two faculty offices housed in the herbarium/museum complex. Special collections: Poaceae and Cyperaceae; John W. Thieret plant and book collections. On-going projects: barcoding and databasing of specimens. Contact the curator for loans and visitation. On 16 March, 2006, as part of a celebration of the life and works of John W. Thieret, the KNK was officially named the John W. Thieret Herbarium; Dr. Thieret left his invaluable book collection to the herbarium (about 600 botanical works with an excellent representation of floras for various areas), where it will be retained as a non-circulating reference collection, and will be available for use by visitors to the herbarium.

Curator, Maggie Whitson (Solanaceae, *Physalis*). **Research Associate,** Landon McKinney (*Carex, Viola*).

Lexington

Herbarium, College of Agriculture. Established about 1887. About 21,000 vascular plant specimens. Used primarily for Extension Weed Science activities that involve plant identification and control. Emphasis on agricultural weeds of Kentucky. Special collections: Harrison Garman, Mary Didlake, and Patricia Haragan.

Curator, J. D. Green.

Lexington

Herbarium of the University of Kentucky (KY). Reestablished in 1948 after fire destroyed the previous collection. 59,300 total plant specimens. Elmer's Glue-All® Multi-Purpose Glue; no insect repellent used; 0°F for 5 days for freezing regime. One student worker per semester; departmental funds average $500/year. Herbarium cabinets number 45, no compactors, in 640 sq. ft. room. On-going projects: Floracliff; Griffith Woods; McCreary/Whitley Counties. One year loans, visitors welcome by appointment. The herbarium has recently been moved from the Forestry Building to the Insectory/Conservatory Building, which has notable improvements, including a better roof, interior, and air conditioner/climate control.

Curator, Robert Paratley (secondary chemistry & medicinal plants; anthropogenic influences on forests).

Louisville

The University of Louisville has recently decided to transfer the Davies Herbarium (DHL) to the Western Kentucky University Herbarium. In 1995 it contained about 30,000 specimens (for the current survey these numbers are considered to be part of the WKU collection). A small set of specimens will be retained in 6 to 8 cabinets by the Biology Department for teaching purposes (W. S. Davis pers. comm.).

Morehead

Herbarium of Morehead State University (MDKY). Established in 1930s. 26,718 total plant specimens. White glue adhesive; naphthalene as insect repellent; no freezing regime. One student worker per semester (or year); departmental funds none. Herbarium cabinets number 22, no compactors, in a 646 sq. ft. room. On-going projects: pteridophytes, bryophytes, and lichens of Carter Caves. Open to qualified researchers.

Curator, Allen C. Risk (bryophytes and lichens).

Murray

Herbarium of Murray State University (MUR). Established in 1967. 43,647 total plant specimens. Microsoft Access and Index Kentuckiensis used for databasing. Elmer's Glue-All® Multi-Purpose Glue; naphthalene insect repellent; minus 20°C for 3 days for freezing regime. Two student workers per semester; departmental funds average $150/year. Herbarium cabinets number 31, no compactors, in a 600 sq. ft. room. Special collections: Raymond Athey collections and herbarium collection at Hancock Biological Station, which is part of MUR. On-going projects: preparing to relocate to new biology building in 2008. Loans to recognized herbaria; visitation during business hours or by appointment.

Curator, Dayle Saar (systematics, population genetics of rare and endangered species,

and phylogeography); **Web Development,** Matthew Williamson.

Herbarium, Hancock Biological Station, Murray State University. As a part of MUR, the Hancock Biological Station has 1200 mounted specimens in 3 cabinets from the published floras of Hancock Biological Station and the Elk and Bison Prairie from the Land Between the Lakes National Recreation Area for teaching, research, and reference purposes. These specimens are databased in Microsoft Access and the complete list is available on the Hancock Biological Station website.

Information person, Ralph L. Thompson, Berea College.

Richmond

Herbarium of Eastern Kentucky University (EKY). Established in 1974. 70,500 total plant specimens. Microsoft Access and Index Kentuckiensis used for databasing. Elmer's Glue-All® Multi-Purpose Glue; no regular use of insect repellent; 3 days at minus 40°C for freezing regime. Three or four student workers per semester; departmental funds average $1200/year. Herbarium cabinets number 45, no compactors; in a 950 sq. ft. room, plus a second room of similar size for processing activities and office space. Special collections: county floras (graduate student projects), collections of Mary Wharton, Raymond Athey, E. T. Browne, and the Kentucky State Nature Preserves Commission. On-going projects: floristic studies of selected sites from across Kentucky. Loan and visitation policies, standard procedures. A new science building is planned that will include expanded facilities for the EKU Herbarium, with compactors, better climate control, and offices for curators and staff.

Curator, Ronald L. Jones (floristics of Kentucky); **Associate Curators,** Ross C. Clark (floristics of Kentucky, woody plants, systematics of *Ilex,* biogeography), David A. Eakin (bryophytes), and Timothy J. Weckman (floristics of Kentucky, systematics of *Viburnum*).

Williamsburg

Herbarium of University of the Cumberlands. Established in 1985. 3509 total plant specimens. Microsoft Access and Index Kentuckiensis used for databasing. White glue adhesive; naphthalene insect repellent; no freezing regime. One student worker per semester; departmental funds average less than $100/year. Herbarium cabinets number 6 full-size and 2 half-size, and one drying cabinet, no compactors, in a 320 sq. ft. room. Loans made with letter of request; visitors welcome but advance notice appreciated.

Curator, Todd Yetter (Lamiaceae, Bahamas, and wetland species).

Wilmore

Herbarium of Asbury College. Established in 1967. 3505 total plant specimens. Elmer's Glue-All® Multi-Purpose Glue and herbarium paste; naphthalene as insect repellent; no freezing regime. No student workers; some funds available for basic supplies. Herbarium cabinets number 2 full-sized and 1 half-sized, within a botany lab of about 900 sq. ft. Loan availability limited, but visitors welcome. Plans are underway for a major renovation of the building that presumably will provide better housing and climate control for the herbarium.

Curator, Ann Witherington.

RESULTS AND DISCUSSION

Total collections of vascular and nonvascular specimens in the state now number 338,100. Vascular plants number 324,700, while nonvascular plant specimens number about 13,400. Collections of vascular and nonvascular plants at the five regional universities total 231,500 (68% of the total), while collections at the private colleges (with the great majority at Berea College) totaled 26,300 (8%) and collections at the University of Kentucky total 80,300 (24%).

The current total of 338,100 herbarium specimens represents an increase of 92,000 or 37% since the article by Jones et al. (1995). Herbaria are reported for 12 institutions, while the 1995 article reported herbaria for 13 institutions (the missing institution is the Davies Herbarium at the University of Louisville). The University of Kentucky showed an increase in collecting activity, adding nearly 8,000 specimens in the last decade. The five regional universities nearly doubled their vascular plant collections, from 121,500 to 220,000, with the greatest increases at Eastern Kentucky University and Western Kentucky University. Nonvascular collections at the re-

gional universities rose from 1100 to 11,900, with the great majority of increase at Morehead State University. Collections at private institutions, with the exceptions of Berea College and the University of the Cumberlands, remain about the same as in the previous survey.

Botany professors with curatorial duties have been hired at seven of the twelve institutions since 1995, with veteran curators remaining only at Berea, Eastern Kentucky University, University of the Cumberlands, and the two University of Kentucky facilities. Another major change is the closing of the Davies Herbarium at the University of Louisville and the transfer of most collections to Western Kentucky University. This herbarium was founded in the 1930s by H. B. Lovell and associates and later became in the 1950s and early 1960s, under the direction of P. A. Davies, the most active botanical research facility in the state. This abandonment of natural history collections at major institutions has become, sadly, a nation-wide trend, as was noted in Jones (2005).

Other changes include an increased interest in databasing. In 1995, only two facilities were actively engaged in databasing, and now that number has increased to four (WKU, EKY, MUR, and University of the Cumberlands). A total of 75,900 specimens are now databased in Kentucky.

The total number of herbarium specimens in Kentucky is similar to that of West Virginia but is far below those reported for Tennessee and Ohio based on Holmgren and Holmgren (1998): Tennessee with 700,000 at 10 institutions, not including 300,000 more that have been transferred from Vanderbilt University (VDB) to the Botanical Research Institute of Texas (BRIT); Ohio with 1,600,000 at 14 institutions; and West Virginia with 300,000 at 9 institutions. The discrepancy is even greater for nonvascular plants, as previously reported in 1995.

It is apparent, that although significant strides have been made, Kentucky still has much work to be done in gathering sufficient knowledge on the occurrence and distribution of plant species. New state records are still being found on a regular basis, and, more significantly, species occurring in Kentucky and new to science have been discovered at an average rate of one per year for the last 25 years (Jones 2005). Many areas of the state, in particular the counties in eastern and south-central Kentucky, have received little floristic attention. Many counties are known to be reservoirs of high botanical biodiversity, but remain largely unexplored. Furthermore, many of these areas are under severe threats from logging, mining, development, and other activities. Today in Kentucky over 130 acres of forested lands per day are being lost to urban areas and roads (Kentucky Division of Forestry 2004).

It is hoped that Kentuckians will recognize the value of preserving their rich botanical heritage, that our institutions will continue to hire field-oriented botanists and that they will work to maintain active herbarium collections to document and help preserve these diminishing resources.

ACKNOWLEDGMENT

The cooperation of all those who responded to the survey is deeply appreciated.

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The Beta-actin Gene in Esocids and Independent Evolution of the Actin Gene Family Members

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ABSTRACT

The five species in the genus *Esox* are popular gamefish that form a unique phylogenetic group based on their characteristic duckbill morphology as well as recent molecular data. To better understand the interspecific relationships in this genus, the beta-actin gene was isolated from each of the five species, and sequence comparisons were carried out for nucleotide and amino acid positions within the gene. Additionally, an internal region of each actin gene family member was sequenced from *Esox masquinongy*. In contrast to published reports on *Takifugu rubripes*, where nine actin genes were identified, only six actin genes were detected in the esocids. The similarity and differences between these family members is outlined and the evolution of the actins is discussed. The significant intronic divergence between the actin family members among the five *Esox* species makes multi-actin gene amplification by polymerase chain reaction (PCR) a potential diagnostic marker for species within a genus, and suggests its use in hybridization or population studies as an alternative to other DNA fingerprinting techniques.

KEY WORDS: Esox, actin, gene evolution

INTRODUCTION

The cytoplasmic beta-actin gene encodes a structural protein associated with the cytoskeleton and is highly conserved across taxa at the amino acid and nucleotide level. The conservation level precludes use of the amino acid sequence for interspecific phylogenetic assignment, and even the exonic nucleotide sequences lack a desirable level of divergence. However, character features of the introns (e.g., substitutions, insertions and deletions) provide meaningful information useful at all levels of phylogenetic assessment. In addition, the gene is so ubiquitously present across phyla that it provides a link between distantly related organisms and has been used previously for this reason (Bhattacharya and Weber 1997; Baldauf et al. 2000; Carlini et al. 2000; Goodson and Hawse 2002; Hwang et al. 2002).

Beta-actin is a member of a multi-gene family that typically includes six members in vertebrates (Vandekerchkove and Weber 1978; Venkatesh et al. 1996). The functions of these genes range from cytoskeletal structure to the muscle actins, and the amino acid conservation between members is very high. Most of the members are virtually indistinguishable by sequence analysis alone but show distinct patterns of expression across tissues (Venkatesh et al. 1996). Beta-actin is distinct from its counterparts by having several signature amino acid substitutions at the termini that permit its identification as well as providing a tool for isolation by polymerase chain reaction (PCR) amplification. Between distinct organisms, such as land vertebrates and teleost fishes, the conservation at the nucleotide level is as high as 90% (Liu et al. 1990). Because of its predictably constant expression as a cytoskeletal protein, it is frequently exploited as a control in gene expression or protein assays.

In this study, I utilized the actin genes to examine differences in *Esox* (Esocidae), which comprises five fish species with characteristic duckbill morphology. This genus has been the focus of recent phylogenetic examinations to determine the relationships among the esocids and their close relatives the umbrids (mudminnows) and salmonids (salmon, trout and char) (Grande et al. 2004). In these studies, morphological character matrices were compared with comprehensive molecular data including nuclear and mitochondrial genes (López et al. 2000; López et al. 2004). As with other studies, one interesting conclusion is that historical hybridization events have obscured the meaning of sequence characters found in the mitochondrial genome (López et al. 2004). This has directed attention to nuclear genes and the importance of finding addi-

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tional nuclear genes useful in broad studies, in the same manner as RAG-1, growth hormone, and other genes that can be reliably amplified from diverse taxa with conserved primers. In this work I examine some of the intron characteristics of the beta-actin gene as a possible phylogenetic marker and the unique and independent evolution of gene structure in the actin family members in *Esox masquinongy.* The whole gene sequence is assessed for its utility in producing the correct phylogenetic relationships, and the possible applications of simultaneously amplifying the multiple actins from these species is discussed.

MATERIALS AND METHODS

Specimens

Tissue and DNA samples from *Esox* used in the molecular analyses are stored in the collection at Morehead State University (MOSU), except those obtained as gifts. The following specimens were used to obtain sequence data: *Esox masquinongy,* Minnesota Muskie Farm, Inc. (Alexandria, MN; MOSU2329); *E. lucius,* Rainy Lake, Ontario, 131-Gut-01, gift from M. Butler; *E. niger,* St. John River, New Brunswick, 135-NBR-01, gift from M. Butler; *E. reicherti* and *Novumbra hubbsi,* gift from A. López; *E. americanus,* McCracken County, KY (MOSU2326). Sunfish DNA was taken from *Lepomis cyanellus* and *L. macrochirus* specimens collected from South Elkhorn Creek in Woodford County, KY, and from Town Branch in Fayette County, KY. DNA from these specimens is in the author's possession and is available upon request.

Isolation of Genomic DNA

Small fin clip biopsies (5 mm^2) were incubated in 250 µl lysis buffer (100 mM Tris, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) and 5 µl proteinase K (from a 100 µg/ml stock solution) at 52 °C for 2 hours. Samples were vortexed and centrifuged in a microfuge for 8 minutes at top speed. The supernatant was then transferred to a fresh tube containing 400 µl isopropanol. The tube was inverted 10 times and centrifuged for 1 minute at top speed, and the supernatant was removed and discarded. The pellet was washed with 95% ethanol, dried briefly, then resuspended in water.

Cloning of Beta-actin Genes

Beta-actin whole gene primers were designed to anneal to the first 9 and the last 9 codons of the cytoplasmic beta-actin gene based on the conserved sequences of several fishes (Liu et al. 1990; Venkatesh et al. 1996; Hwang et al. 2002). The polymerase chain reaction was carried out with each sample using the following amounts: 5 µl genomic DNA (from a 10 ng/µl stock), 1.5 µl of each primer (from a 10 ng/µl stock), 12.5 µl of 2× GoTaq PCR master mix (Promega), and water up to 25 µl. The whole gene forward primer (BAF) sequence is 5′ atg gat gat gaa atc gcc gca ctg gtt 3′; the whole gene reverse primer (BAR) sequence is 5′ tta gaa gca ttt acg gtg gac gat gga 3′. Cycle parameters were 95 °C for 15 minutes, followed by 30 cycles of 95 °C for 30 sec, 47 °C for 30sec, 72 °C for 1 minute. The genomic fragment produced from each fish was approximately 2 kb and was cloned into the pGEM-T Easy vector (Promega). One or more clones from each species were sequenced and compared with known beta-actin genes for verification, and the exons were determined using AG/GU consensus splice sites and the virtually invariant beta-actin amino acid sequence. Sequencing was performed by MWG Biotechnology (High Point, NC) using primers that span the region of the gene. The sequencing primers are Beta1 5′ gcc cag agc aag aga ggt atc ctg acc ct 3′; Beta2 5′ gag acc ttc aac acc ccc gcc atg tac gt 3′; Beta3 5′ cag gtc atc acc atc ggc aay gag agg tt 3′; Beta4 5′ gtg ttg gcg tac agg tcc tta gcg atg tc 3′; Beta5 5′ agg atc ttc atg agg tag tct gtg agg tc 3′; Beta6 5′ tac ctg ggt cat ctt ctc cct gtt gg 3′. The data for *Lepomis cyanellus* were taken from a previous study (Peyton 2004). Sequences were compiled and analyzed using the Vector NTI software package. Alignments of beta-actin sequences were performed with the AlignX (ClustalW) application.

Amplification of Actin Genes with Internal Primers

The remaining members of the gene family were subcloned by selecting plasmids that contained inserts for each of the PCR fragments produced using the Exon 3 Forward (EX3F) and Exon 5 Reverse (EX5R) primers. These primers were designed around se-

Table 1. Nucleotide lengths for beta-actin introns and exons in the species examined. Lengths for *Takifugu rubripes* taken from the beta-actin 1 gene (Accession no. U37499; Venkatesh et al. 1996).

	Exon 1	Intron 1	Exon 2	Intron 2	Exon 3	Intron 3	Exon 4	Intron 4	Exon 5	Total
Takifugu rubripes	123	88	240	353	439	70	182	77	144	1716
Esox masquinongy	123	319	240	415	439	93	182	315	144	2270
Esox lucius	123	265	240	404	439	94	182	270	144	2161
Esox reicherti	123	272	240	395	439	94	182	281	144	2170
Esox niger	123	277	240	405	439	91	182	301	144	2202
Esox americanus	123	300	240	402	439	90	182	292	144	2212
Novumbra hubbsi	123	333	240	403	439	87	182	123	144	2074

quence from *T. rubripes* that was conserved among all actin members on exons 3 and 5 (Venkatesh etal. 1996). The EX3F sequence is 5′ ttc cgt tgc cca gag gcc ctc ttc cag 3′; the EX5R sequence is 5′ tcc tgc ttg ctg atc cac atc tgc tgg 3′. Polymerase chain reaction was carried out as before using these cycle parameters: 95 °C for 2 minutes, followed by 30 cycles of 95 °C for 30 sec, 47 °C for 30 sec, and 72 °C for 2 minutes. All samples were sequenced by MWG Biotechnology as stated above.

RESULTS

The beta-actin genes from each *Esox* species were isolated using consensus oligonucleotides (BAF and BAR) that anneal to the first and last nine codons. These primers amplify a region of beta-actin containing five exons and four introns of the gene, including the entire amino acid reading frame. In this manuscript, the amplified exons are labeled one through five with distances measured from the start codon to the stop codon. In other published reports, such as that for *Takifugu rubripes,* there are several additional nucleotides upstream of the start codon and an additional untranslated exon 5′ to the start codon (Venkatesh 1996). Two additional beta-actin genes were used for comparison: the published sequence from *T. rubripes* (Venkatesh et al. 1996) and the beta-actin sequence from *Novumbra hubbsi* (determined in this project, not published), which is the most closely related species to the *Esox* genus (López et al. 2004).

The comparison of nucleotide counts for each exon and intron examined is shown in Table 1. No differences were observed in the lengths of the coding regions, which is consistent with the high level of conservation in the amino acid sequence across taxa. Within the genus, the two sister-species groups (López et al. 2004) are sorted together by nucleotide number alone: (1) *Esox lucius* and *E. reicherti* which differ by only nine nucleotides; (2) *E. niger* and *E. americanus* which differ by only ten nucleotides. All of the esocids, as well as *Novumbra hubbsi,* are significantly larger (445–554 nucleotides) than *Takifugu rubripes* which is known to have a compact genome. The outgroup species, *N. hubbsi,* differs significantly in length from the esocids only because of an unexpectedly short fourth intron, which is less than half the length of the smallest *Esox* fourth intron.

To address the number of actin genes present in the esocid genomes, as well as their relative divergence from the putative common ancestral gene sequence, two internal primers were designed that would anneal to internal exons and amplify two partial exons, one complete exon, and two introns. The products of this amplification represented a region spanning exons 3, 4, and 5 in our naming scheme and are shown for each of the *Esox* species as well as two *Lepomis* positive controls (Figure 1). At a restrictive annealing temperature, five or six products were reliably produced for each species and ranged in size from approximately 500–1000 bp. The nature of these products was investigated by choosing one species, *Esox masquinongy,* and subcloning and sequencing each product. Sequence alignment and analysis were performed with the Vector NTI software package. Comparison with the amino acid sequences of actin genes in *Takifugu rubripes* identified putative coding regions and splice junctions for six different actin genes. The relative sizes of the intron lengths for each amplicon (Figure 2), showed the unequal expansion of the non-coding regions since divergence from the ancestral actin

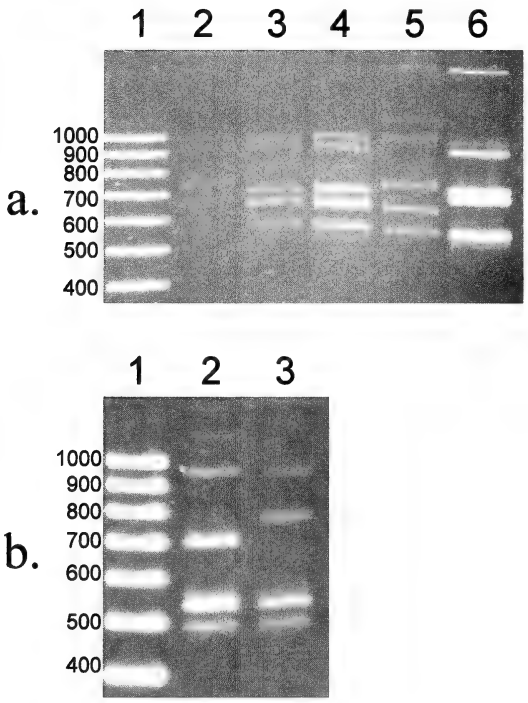

Figure 1. The products from PCR amplification using internal primers that anneal to the actin gene family members. Panel 1a shows the products from the five esocid species: lane 1, size standard; lane 2, *Esox masquinongy*; lane 3, *E. lucius*; lane 4, *E. reicherti*; lane 5, *E. niger*; lane 6, *E. americanus*. Panel 1b shows the product from two *Lepomis* sunfish: lane 1, size standard; lane 2, *Lepomis macrochirus*; lane 3, *L. cyanellus*. The molecular weight ladder contains seven bands from 400 bp to 1000 bp.

gene. The hatched box in the 920 bpproduct represents an exact 17 bpinverted repeat, separated by a five nucleotide spacer: AAACTT GCATAACAGTA *TTTAC* TACTGTTATGCA AGTTT. The function of this structural motif is not known, but the intrastrand basepairing possibilities suggest that the RNA would be capable of a highly stable stem-loop structure at this location.

The putative amino acid coding sequence, separated into triplet codons and aligned, is shown in Figure 3A. The majority of sequence divergence occurs at the third position in each codon, which includes variation in the first splice site (indicated by arrows). The second splice site, joining exon 4 and 5, is perfectly conserved across the six products and represents a closer fit to a consensus splice sequence. Across the length of the examined region there are six positions where amino acid substitutions occur (asterisks in Figure 3B). The 500, 550 and 1000 basepair (bp) fragments are indiscernible from each other at the amino acid level. Compared with their amino acid sequence, the 700 and 920 bp sequences have one substitution each (Alanine to Threonine, third asterisk), occurring at the same location as an Alanine to Threonine substitution in the alpha-skeletal 2 and alpha-anomalous actin in *Takifugu rubripes* (Venkatesh et al. 1996). The 750 bp product contains six substitutions, which identify it as beta-actin when compared to a consensus vertebrate beta-actin protein sequence.

DISCUSSION

The usefulness of molecular data in reconstructing phylogenetic relationships depends on having DNA sequences that show some conservation between taxa but not so much similarity that differences cannot be characterized and recorded. The beta-actin gene has been used in many studies of higher order taxa, but the level of conservation in the coding regions is expected to be above the threshold for deducing intrageneric relationships (Baldauf et al. 2000).

Analysis of the beta-actin sequence from the five esocids indicates that the coding regions for these fish will not be informative, but the introns do contain characters that will assist in assignment of these fish. A rigorous assessment of how well the introns will predict relationships within *Esox* will require beta-actin sequence from multiple specimens of each species, as well as from the umbrids and salmonids. An alignment of the sequence generated in this study, from one specimen of each esocid (with *Novumbra hubbsi* as an outgroup), produces a tree (Figure 5) consistent with all previous data (López etal. 2004). Though not comprising a dataset large enough to answer all phylogenetic questions, the data here are appropriate for a discussion of the structure of the gene and its relationship to other known actin genes.

Within *Esox,* there is little divergence among the lengths of introns and no differences in exon length, in the beta-actin gene. When one looks outside the genus to a phylogenetically close relative, *Novumbra hubbsi,* there is some noticeable difference in intron

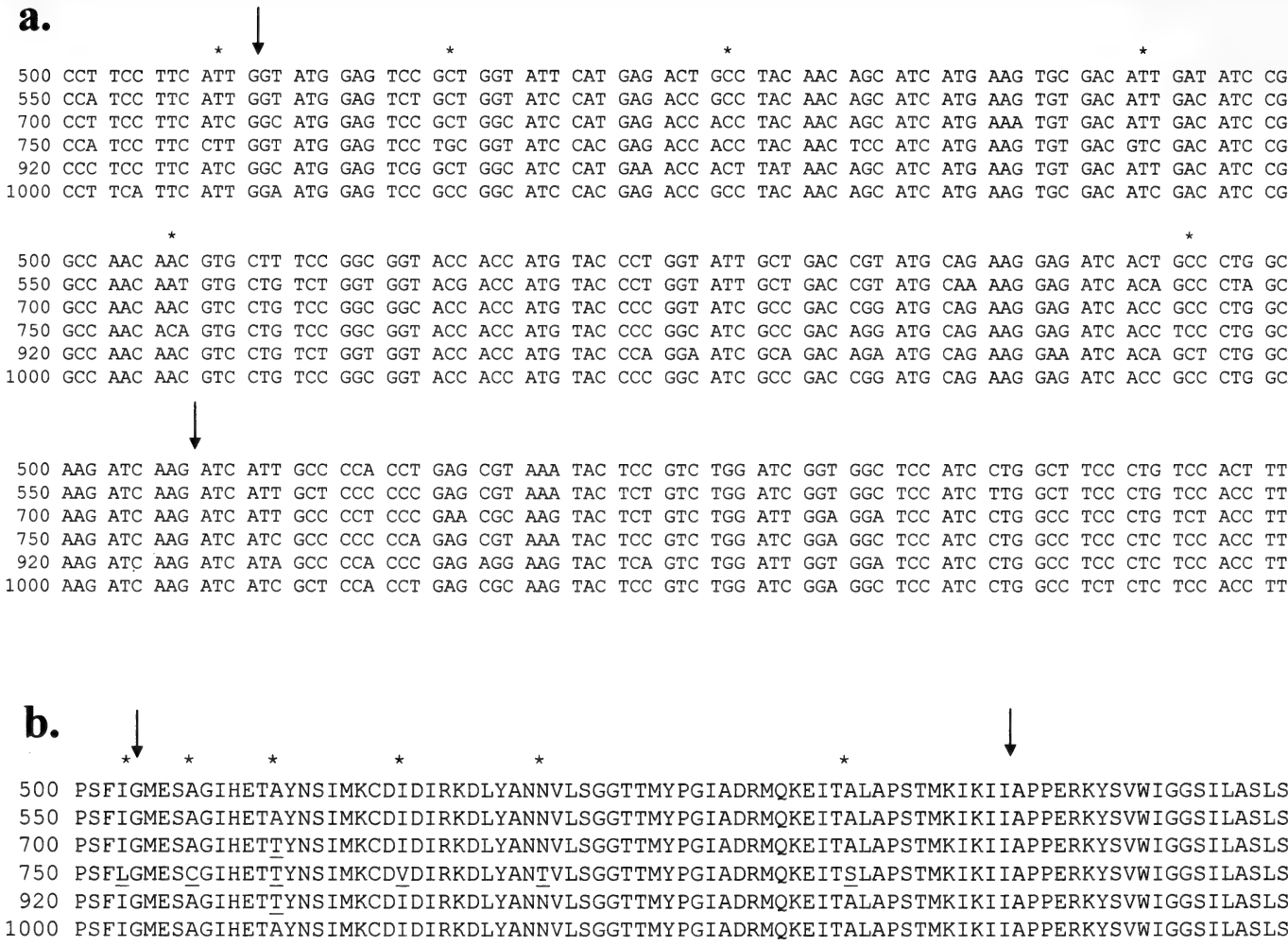

Figure 2. Diagram of the approximate length proportions between exons and introns of the six amplified products from *Esox masquinongy*. Shaded boxes represent exons, lines represent introns, and the hatched box represents a 17 bp inverted repeat. The scale for 100 bp is represented by the bar at the top of the panel.

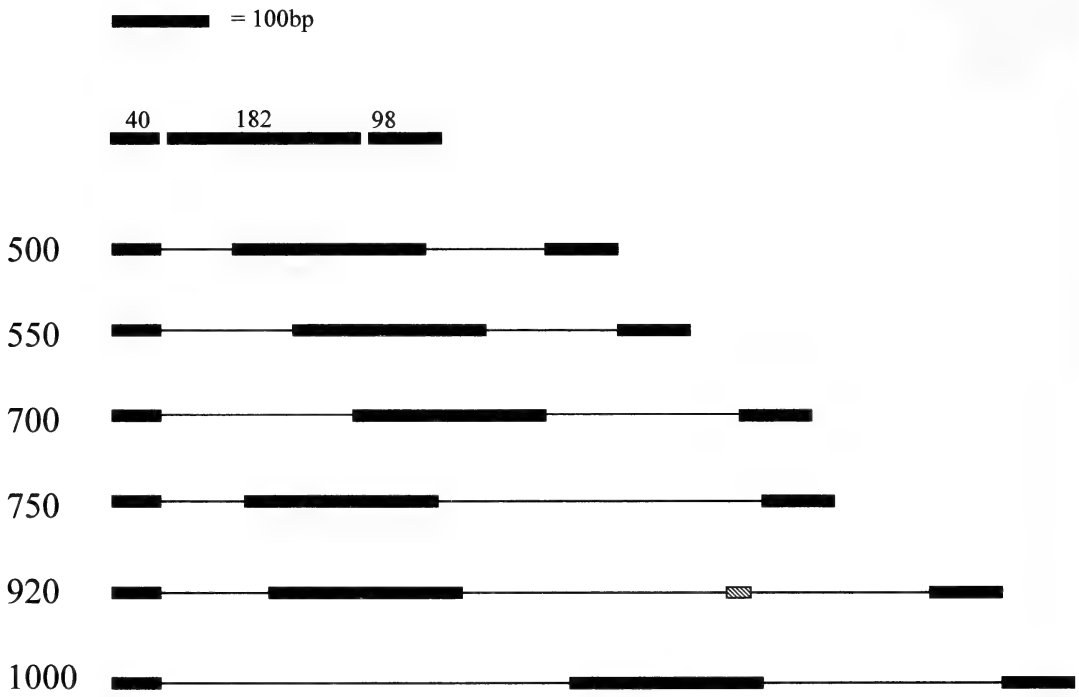

Figure 3. Nucleotide and amino acid alignments of the sequenced products from *Esox masquinongy.* Numbers 500, 550, 700, 750, 920, and 1000 correspond to the approximate basepair length of the PCR product. Splice junctions are indicated by arrows, and locations of amino acid substitutions are indicated by asterisks in each panel. Panel 3a shows the alignments of the reading frames, in triplets, of the partial exon 3, full exon 4, and partial exon 5 of each product isolated. Panel 3b shows the alignment of the amino acids for the same region, with substitutions underlined.

size, particularly in the fourth intron. As more distant taxa are examined, such as *Takifugu rubripes,* the differences in intron length become substantial across all of the introns.

The evolution of intron size and placement become more intriguing when multiple actin family members are brought into the analysis. First, within one species such as *Esox masquinongy,* it is clear that after duplication events increased the copy number of the ancestral actin gene, constraints on intron size and characteristics were not strong enough to retain similarities among the gene family members. Functional constraint on the diverse protein products was significant enough to retain a high degree of conservation at the amino acid level, but there were no similarities in the intron nucleotide sequences examined. One readily identifiable motif, the 17 bp inverted repeat in the 920 product, was not observed in any of the other amplicons.

The amplification protocol used had a sufficient extension time to generate products much greater than 2000 bp in length, but only products in the range of 500–1000 bp were detected for the esocids. When mammalian DNA from rodents and canines is used, a similar number of products are present but are much smaller in length (data not shown). Products in excess of six discrete bands were

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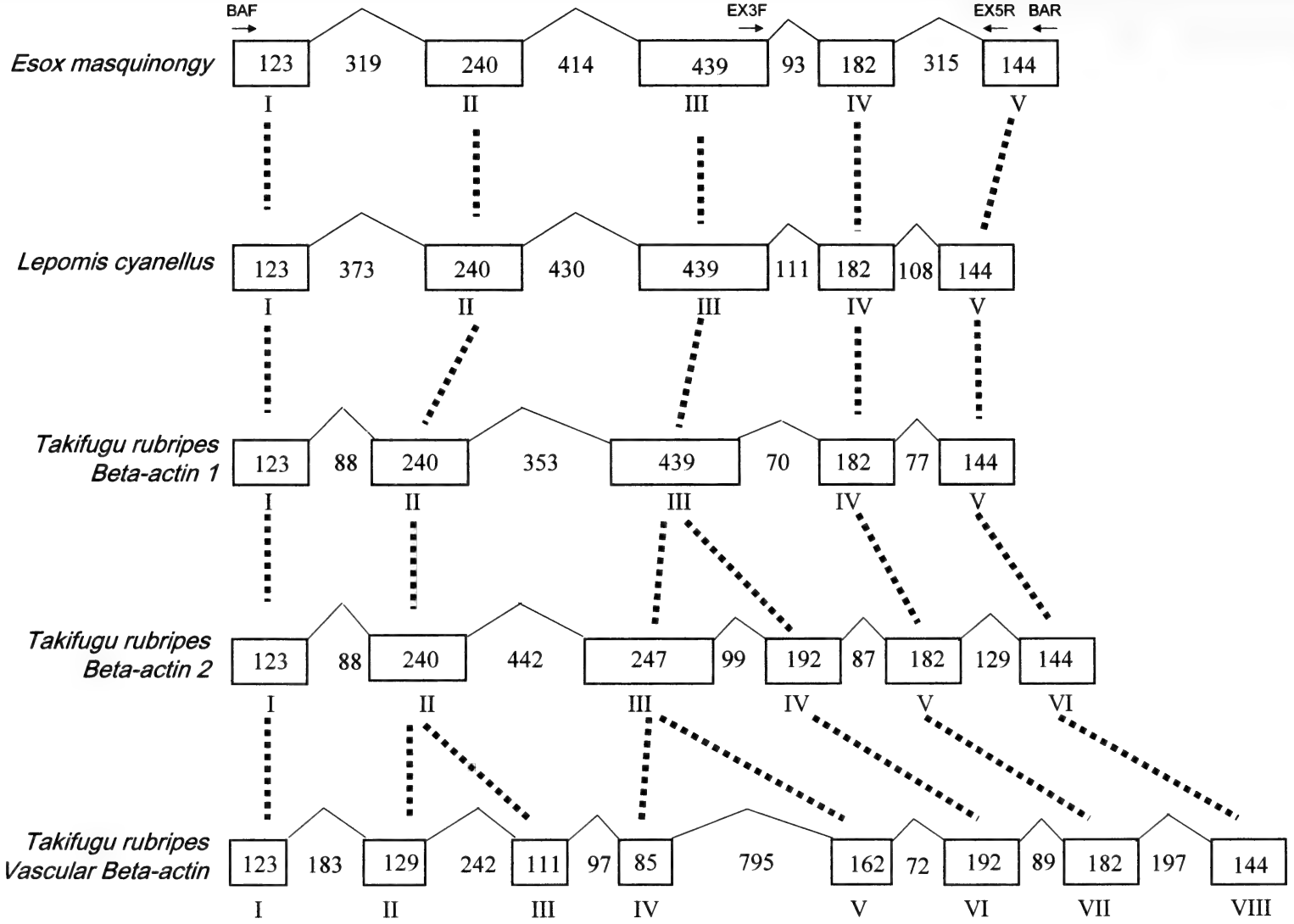
Figure 4. Corresponding exons between the beta-actin genes from *Esox masquinongy, Lepomis cyanellus,* and *Takifugu rubripes.* Exons are represented by boxes, intron lengths are between boxes, and dashed lines show the corresponding exons between species or isoforms. The accession numbers for the *T. rubripes* genes used here are U37499 (beta-actin 1), U38848 (beta-actin 2), and U38850 (vascular beta-actin) (Venkatesh et al. 1996). Approximate primer locations are shown above the gene for *E. masquinongy.*


BAF
EX3F
EX5R
BAR
Esox masquinongy
123
319
240
414
439
93
182
315
144
I
II
III
IV
V
Lepomis cyanellus
123
373
240
430
439
111
182
108
144
I
II
III
IV
V
Takifugu rubripes
Beta-actin 1
123
88
240
353
439
70
182
77
144
I
II
III
IV
V
Takifugu rubripes
Beta-actin 2
123
88
240
442
247
99
192
87
182
129
144
I
II
III
IV
V
VI
Takifugu rubripes
Vascular Beta-actin
123
183
129
242
111
97
85
795
162
72
192
89
182
197
144
I
II
III
IV
V
VI
VII
VIII

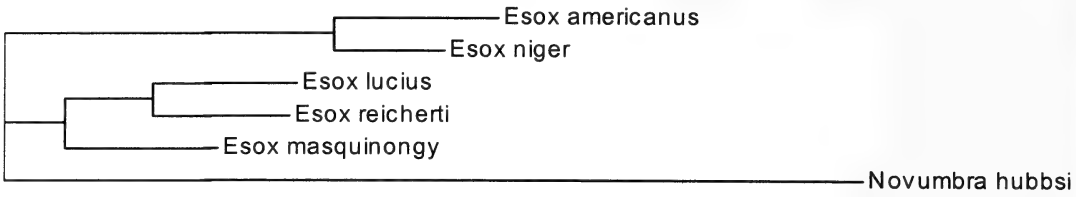


Figure 5. Tree produced by ClustalW analysis of the full length beta-actin gene sequence from all five *Esox* species and *Novumbra hubbsi*.

not observed in the species examined. The possibility that the esocids, or any of the species assayed, could have more than six actin family members cannot be ruled out. However, it is interesting that *Takifugu rubripes*, which was chosen as a model organism by Sydney Brenner because of its compact genome, might have additional isoforms not present in other vertebrates (Venkatesh et al. 1996). Venkatesh et al. (1996) suggest the possibility that two of the *Takifugu* actins were pseudogenes; however. the genes lack the characteristics normally associated with pseudogenes.

The three beta-actin genes identified in *Takifugu rubripes* each carry the same length reading frames (coding for 375 amino acids), but contain alternate numbers of introns (Figure 4, compared with *Esox masquinongy* and *Lepomis cyanellus*). One theory proposed for actin evolution states that a primordial actin gene had eight introns, which were lost during the expansion of the family into multiple members (Carroll et al. 1986). The reverse transcription and re-insertion of partially processed mRNA back into the genome could account for this loss of introns and explains the occurrence of the multiple isoforms. Beta-actins containing two or three additional introns were not detected during isolation of the esocid genes, but their potential existence cannot be dismissed. The form of the gene isolated in this study corresponds to "beta-actin 1," based on intron location and number. The derivation of this structure (five coding exons, four introns) from the vascular beta-actin and beta-actin 2 can be clearly seen as the consolidation of exons II, III and IV, V (vascular beta-actin) and III, IV (beta-actin 2).

The divergent nature of the multi-gene set provides an opportunity to use a genetic test to distinguish species or hybrids. In the case of esocids, natural morphological differences provide an effective means to identify hybrids, which rarely occurs. One artificially produced hybrid, the tiger muskie (*Esox masquinongy* × *E. lucius*), has a distinct color pattern that cannot be mistaken for either parent species. However, there are other intrageneric hybrids that occur frequently in nature, such as between *Lepomis* sunfish species. As shown here, the pattern of actin amplicons is distinctive for the two sunfish sampled. The possibility of using actin multi-gene amplification to discriminate between all twelve species and their hybrids is currently under investigation.

In this study, the beta-actin genes from all five species of the *Esox* genus were isolated and characterized by intron/exon structure. Using primers from internal actin exons, six isoforms from one species were sequenced and determined to be members of the actin gene family. The divergence of intron lengths among the isoforms was species-dependent and may serve as a useful phylogenetic tool for this or other genera. The application of the nucleotide sequence of beta-actin as a phylogenetic marker will be determined when more data from esocids and related teleost fish (umbrids, salmonids) are sampled in future work.

ACKNOWLEDGMENTS

I would like to thank Ben Brammell, Rob Hopkins, and David Eisenhour for their assistance in the collection of specimens. I would also like to thank Andres López for discussion on the *Esox* actin genes and tissue samples and Michael Butler for his kind gift of *Esox lucius* and *E. niger* tissues. This work was supported by a grant from the Institute for Regional Analysis and Public Policy at Morehead State University.

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Evaluation of *Cinygmula* (Ephemeroptera: Heptageniidae) Drift Behavior as an Indicator of Aqueous Copper Contamination

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ABSTRACT

We designed an *in situ* assay for investigating macroinvertebrate drift behavior in response to a point source of environmental pollution. As a model, we used *Cinygmula* nymphs to assess both the presence and level of copper contamination in a stream that was polluted with effluent from an abandoned copper mine. The study showed that *Cinygmula* exhibited increased tendency to drift with increased exposure to aqueous copper. *Cinygmula* drift behavior exhibited graded responses to increasing concentrations of aqueous copper up to and including 78 ppb. The results indicated that a simple yet sensitive *in situ* bioassay could be used to detect environmentally important levels of copper contamination in Haggarty Creek.

KEY WORDS: aqueous copper, *Cinygmula,* in situ bioassay, pollution, drift behavior

INTRODUCTION

Standard laboratory-based, dose-response studies for acute toxicity are often used to determine how some of the aquatic biota respond to specific pollutants (e.g., Dobbs et al. 1994). However, dose-response studies may not take into account sub-lethal effects of some pollutants, especially in a field setting. Drift behavior of benthic macroinvertebrates may be a more sensitive indicator of how pollution affects the aquatic biota than standard laboratory-based, dose-response tests for acute toxicity because benthic macroinvertebrates may release their grip on the substrate and drift downstream when they detect pollutants that are present in relatively low concentrations (Clements 1999). When that occurs, the populations are reduced or are not present in habitats that otherwise would be suitable.

Benthic fauna have been used as indicators of anthropogenic disturbance in some lotic systems. Ormerod et al. (1987) found a drift response of some dipterans, plecopterans, and ephemeropterans, as well as a crustacean, to acidification and acidification plus aluminum. Hopkins et al. (1989) found a substantial drift response of the ephemeroptean, *Baetis,* to acid conditions. Gerhardt et al. (1998) studied the drift response of a crustacean to toxic discharges of copper when developing an online biomonitoring system. Courtney and Clements (1998) found that percent invertebrate drift in most acidic streams was nine times that of the control and also noted that Ephemeroptera was the only insect order to exhibit a significant drift response. Clements (2004) found concentration–response relationships between macroinvertebrate drift and heavy metal concentrations and indicated these were generally more sensitive to metal concentrations than were structural measures such as abundance and richness. Given these studies, we investigated developing an *in situ* bioassay to assess the effects of copper through the drift behavior of Ephemeroptera.

The study system was Haggarty Creek, which is a high-elevation montane stream in the Sierra Madre Range of southeast Wyoming. Portions of this stream are adversely affected by copper-laden effluent from the Ferris–Haggarty copper mine. The effluent has

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reduced the diversity of benthic macroinvertebrates to just a few copper-tolerant species where it flows into the creek, as well as in downstream reaches (Rockwell 2001).

As the effluent-laden water flows downstream, the diversity of benthic macroinvertebrates gradually increases because water entering Haggarty Creek from uncontaminated tributaries dilutes the copper-laden water, making it increasingly less toxic to benthic fauna (Rockwell 2001). As a result, Haggarty Creek exhibits a longitudinal gradient of aqueous copper concentrations, providing a series of sites at which the influence of varying copper concentrations on benthic macroinvertebrate drift could be evaluated.

Individuals in the ephemeropteran family Heptageniidae were chosen as test subjects because of their sensitivity to aqueous metals (Hickey and Clements 1998; Clements 1999). Further, individuals of this family are usually present in high altitude streams of southeast Wyoming as they have morphological adaptations that allow them to cling to the substrate during spring flows. Our surveys (unpublished data) and Rockwell (2001) indicated that there was an abundance of heptageniid nymphs in the genus, *Cinygmula,* in streams that surround Haggarty Creek; so we used this genus in our *in situ* drift study on Haggarty Creek as well as on Bachelor Creek, an uncontaminated tributary. Given the similarities in elevation, substrate, shade, and flow regime between Haggarty Creek and Bachelor Creek, and given that *Cinygmula* were collected in streams all around the affected reaches of Haggarty Creek, we would have expected *Cinygmula* to be in Haggarty Creek were it not for copper contamination.

MATERIALS AND METHODS

Drift Chamber Development

The presence of bedrock and boulders in the upper reaches of Haggarty Creek prevented us from using the techniques of Kiffney et al. (1997), Clements (1999), or even drift nets to study the drift behavior of *Cinygmula* because the streambed could not be penetrated; these sampling techniques all require that sampling tools be deeply inserted into the streambed. Such conditions are common in western montane streams. As such, we designed and used drift chambers that would lie flat on the streambed.

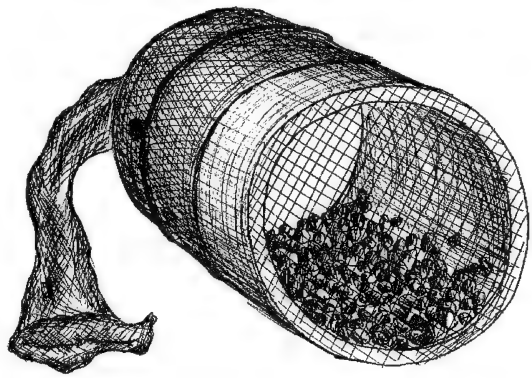

Figure 1. A drift chamber.

Drift chambers (Figure 1) were constructed from 12 cm long and 7.6 cm inside diameter PVC pipes. Chambers were fitted on the downstream end with white nylon leggings, creating catch-bags that extended 10–14 cm when placed in the water. Catch-bags allowed us to easily and unobtrusively count *Cinygmula* that had drifted from the chambers. Plastic screens (2 mm pore size) were permanently affixed to the upstream end of each chamber. Stream substrate particles 2–8 mm in diameter were collected from Haggarty Creek, acid-washed, and secured in a single layer to the inside bottom of each chamber using silicone caulk. This created a substrate of small stones to which *Cinygmula* could cling.

Site Descriptions

Sites on Haggarty and Bachelor Creeks (Figure 2) were chosen for their uniformity of flows, similarity of shady conditions, and differences in aqueous copper concentrations. Specifically, all sites were situated on straight-sided riffles whose flows were within 10 percent of one another; flows were determined using the methods of Gore (1996). We also made sure the sites were situated in the shade to prevent sun-warmed water from causing *Cinygmula* drift. Sites on Haggarty Creek were established so the uppermost (#1) had a high aqueous copper concentration (229 ppb), the middlemost (#2) an intermediate copper concentration (78 ppb) and the lowermost (#3) a low copper concentration (3 ppb)

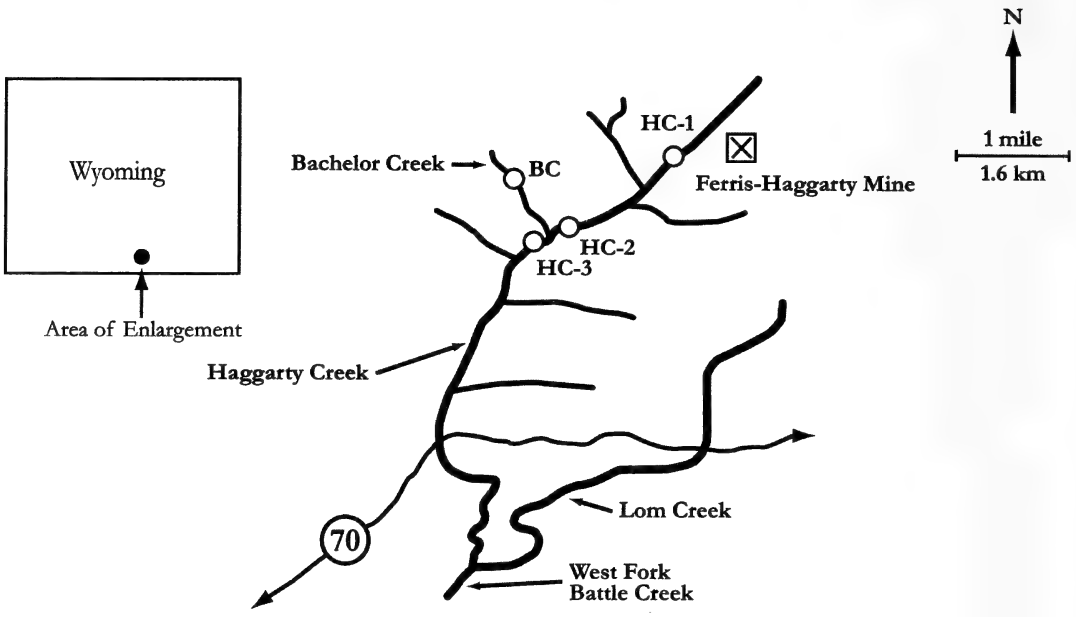

Figure 2. Location of test sites on Haggarty (polluted) and Bachelor (unpolluted) Creeks.

(Rockwell 2001). In contrast, the copper concentration at Bachelor Creek was less than detection limits (<1 ppb) (Rockwell 2001). Sites #1 and 2 on Haggarty Creek and the Bachelor Creek site were on first order streams while site #3 on Haggarty Creek was on a second order stream. All sites had cobble/gravel substrates with moderate slope. Elevations of Haggarty Creek sites #1, 2, and 3 were at 2957, 2865, and 2743 m while elevation of the site on Bachelor Creek was at 2774 m a.s.l.

Cinygmula Collection

Cinygmula were collected from Bachelor Creek, via kick sampling, using a D-net (1 mm pore size). *Cinygmula* were transferred with a glass pipette to a 1-L Nalgene® bottle filled with water from Bachelor Creek; specimens were identified to genus because dichotomous keys to species were unavailable (Ward et al. 2002).

Chamber Loading and Submersion

Chambers were loaded with *Cinygmula* by first placing a PVC cap over their upstream ends. Water from a given site was then added by dipping the chambers into the stream. Next, 10 *Cinygmula* were transferred from the Nalgene® bottle to each water-filled chamber using a glass pipette; no more than 10 *Cinygmula* were placed in a chamber to prevent crowding. After loading, nylon catch-bags were placed over the downstream ends and secured with cable ties. Three chambers were loaded at each site, providing a total of 30 *Cinygmula* per replicate per site.

Before submersion, a second PVC cap was placed over each downstream (i.e., catch-bag) end. Chambers were then submerged, placed on the stream bottom, and anchored with large rocks; chambers were covered by 5 cm of water. After submersion, the *Cinygmula* were allowed to settle on the chamber's substrate for 10 min. The downstream cap then was carefully removed so that a backwash was not created and the catch-bag extended slowly downstream. Finally, the upstream cap was slowly removed to prevent an initial sudden flow through the chamber.

Chamber Monitoring

After submersion, each catch-bag was assessed at three intervals. The first occurred 3 min after the upstream cap was removed (post-settling). Additional assessments were made at 10 and 60 min post-settling (exposure times). At each exposure time, *Cinygmula* found in all catch-bags at a site were counted (drifting *Cinygmula*). The study was first conducted on 30 June 2000, and then repeated

Table 1. Estimated logistic parameters for drift curves developed at each of four stream sites; the Bachelor Creek site was not contaminated with aqueous copper, but all Haggarty Creek sites were, with Haggarty Creek site #1 being the most contaminated and Haggarty Creek site #3 being the least: Sierra Madre Range, southeast Wyoming.

Site	γ^a	α^b	β^c	$\lvert t \rvert$	P_{slope}
Bachelor Creek	1.0	−2.9079	0.011792	1.32	0.1142
Haggarty Creek #1	−0.6	1.3635	−6.6626	7.12	0.0001
Haggarty Creek #2	−0.4	1.5781	−4.2962	5.75	0.0004
Haggarty Creek #3	−0.8	−0.81599	−2.3246	2.32	0.0267

[a] Curve fitting exponent.
[b] Logit *y*-intercept.
[c] Logit slope; values of the logit slope were subjected to *t* tests, for which one-tailed *P* values are provided.

on 1 and 2 July 2000, providing a total of three replicates for statistical analysis.

Statistical Analysis

Response of drifting *Cinygmula* to exposure times was determined through regression analysis. For each site, we regressed the number of drifting *Cinygmula* on exposure times. As the relation between drifting *Cinygmula* and exposure times was not linear, being bound by an asymptote of 30 *Cinygmula* at a site per replicate, we used logistic regression to develop the response curves (drift curves) (Haberman 1978). Specifically, we used the software program, LINLOGIT (Legg 2003), to fit drift curves of the following form:

$$\hat{Y}_i = T/\{1 + \exp(-1(\alpha + \beta X_i^{\gamma})]\} \quad (1)$$

where $\hat{Y}_i$ is the ith fitted value on a curve, T is the total number of *Cinygmula* being tested at a site for a replication (30 in this study), exp is the base of the Napierian logarithm, α is the y-intercept expressed in logit scale, β is the slope also in logit scale, X_i is the ith exposure time (min), and γ is a curve-fitting exponent, the value of which is iteratively derived. The slopes of Equation 1 were then tested for significant departures from zero using t tests, testing the null hypothesis that the logit slope was equal to zero against the alternate that it was less than zero (type I error rate = 0.05) (Haberman 1978); logit slopes less than zero corresponded with positive drift curves. The PROBT function of the Statistical Analysis System (1989) was used to calculate P values. Once drift curves were developed, we compared them, in pair-wise fashion, using the F statistic (Weisberg 1980) to test the null hypothesis that drift curves were the same (equal drift rates) against the alternate that they were different.

RESULTS

Influence of Exposure Times on Drift

Results showed that, for the uncontaminated site on Bachelor Creek, the logit slope was essentially zero (Table 1). Graphically, this appeared as a 'flat' drift curve (Figure 3) and indicated that, in the absence of detectable aqueous copper contamination, the drift behavior of Cinygmula did not increase with increasing exposure times through 60 min.

At the most contaminated site of Haggarty Creek (#1), the logit slope was less than zero (Table 1). Graphically, this corresponded with a drift curve that rose distinctly through the 3 and 10 min assessments but less so between the 10 and 60 min assessments (Figure 3). At the next-most contaminated site of Haggarty Creek (#2), the logit slope was also less than zero (Table 1). Graphically, this corresponded with a drift curve that also rose distinctly through the 3 and 10 min assessments but less so between the 10 and 60 min assessments (Figure 3). At the least-contaminated site of Haggarty Creek (#3), the logit slope was again less than zero (Table 1). Graphically, this corresponded with a drift curve that rose distinctly through the 3 min assessment, but less so between the 3, 10, and 60 min assessments (Figure 3).

Influence of Aqueous Copper on Drift Rates

An overlay of the four drift curves (Figure 4) suggested that the drift rates of *Cinygmula* differed among some sites. Results from F tests indicated that the drift curve from the uncontaminated site of Bachelor Creek was different from that of the least contaminated site of Haggarty Creek (site #3) ($F_{2,14} = 5.12$, $P = 0.0214$). Further, the drift curve from Bachelor Creek was different from the curves

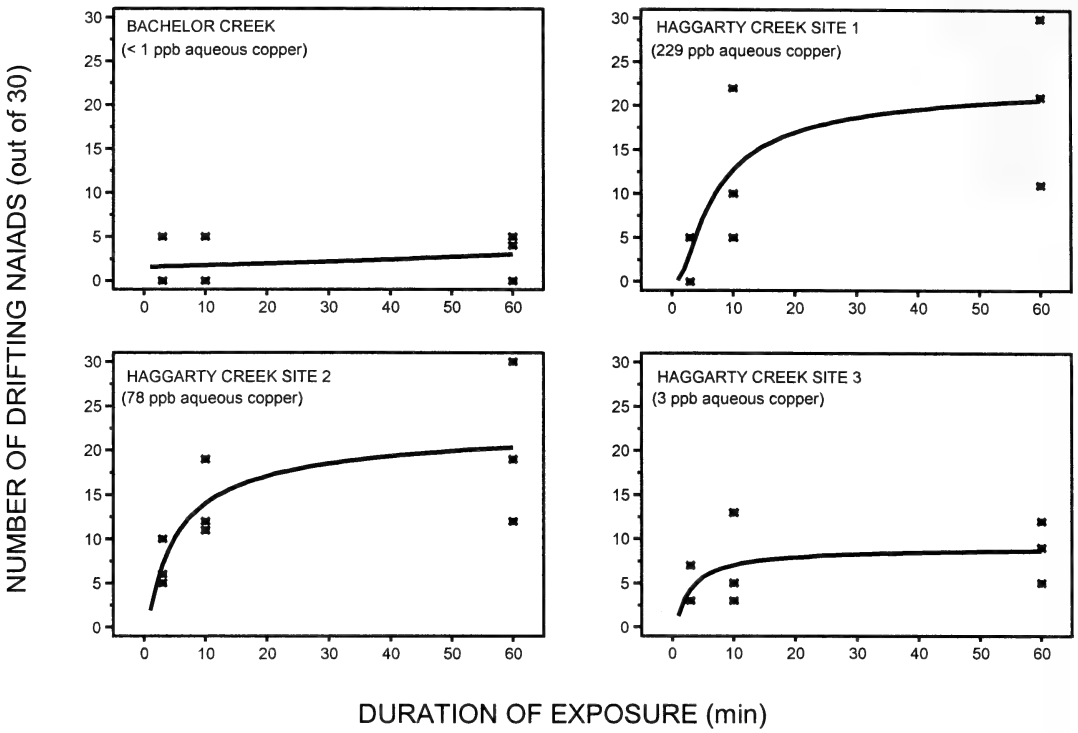

Figure 3. Logistic regression (i.e., drift) curves describing the relation between observed number of drifting *Cinygmula* and exposure times from a site that was uncontaminated by aqueous copper on Bachelor Creek, as well as from the most contaminated site on Haggarty Creek (#1), the next-most contaminated site on Haggarty Creek (#2), and the least contaminated site, also on Haggarty Creek (#3); Sierra Madre Range, southeast Wyoming.

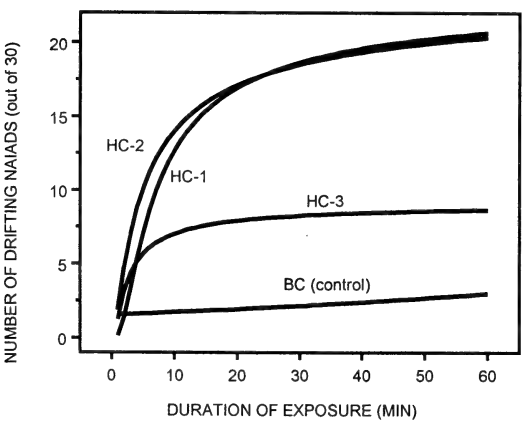

Figure 4. Superimposed drift curves developed from experiments conducted on the most contaminated site on Haggarty Creek (HC-1) (229 ppb aqueous copper), next-most contaminated site on Haggarty Creek (HC-2) (78 ppb aqueous copper), the least contaminated site on Haggarty Creek (HC-3) (3 ppb aqueous copper), and from an uncontaminated site that was located on Bachelor Creek (BC control) (<1 ppb aqueous copper); Sierra Madre Range, southeast Wyoming.

of the more heavily contaminated Haggarty Creek sites #2 ($F_{2,14} = 19.13$, $P = 0.0001$) and #1 ($F_{2,14} = 11.64$, $P = 0.0011$). In addition, the drift curve from the least contaminated Haggarty Creek site #3 was different from the curves of the more heavily contaminated sites #2 ($F_{2,14} = 6.55$, $P = 0.0098$) and #1 ($F_{2,14} = 4.25$, $P = 0.0361$). Drift curves from the two most heavily contaminated sites on Haggarty Creek were the same ($F_{2,14} = 0.2866$ $P = 0.7551$) (Figure 4).

DISCUSSION

The Ferris–Haggarty copper mine was the most productive copper mine in North America at the turn of the 20th century (Rockwell 2001). Mine operations were such that untreated effluent was allowed to flow from the mine into Haggarty Creek. In the 1970s, the Wyoming Department of Environmental Quality instituted new environmental standards that required treating the effluent before it was allowed to flow from the mine. As

the cost for supplying this treatment was not met, the mine became inactive and was abandoned, leaving the untreated effluent flowing into Haggarty Creek (Chervick and Harp 1977; Bell 1996). At present, the affected reaches begin where effluent joins Hagarty Creek and continue downstream about 18 km.

Plans for reclaiming the affected reaches of Haggarty Creek involve studying the bioaccumulation of metals in benthic fauna. This research has been conducted by Rockwell (2001). Plans for reclaiming the affected reaches of Haggarty Creek also have involved studying *in situ* responses of benthic fauna to effluent from the Ferris–Haggarty mine; this research has not yet been conducted.

Drift of some benthic macroinvertebrates is not always due to pollution, as some occurs naturally. For example, Peckarsky and Cowan (1995) found that most Ephemeroptera and Plecoptera exhibited a propensity for nocturnal drift. In another study, Peckarsky (1996) found that the drift of five ephemeropterans was sometimes induced by predators, but a lack of food also increased the drift of *Baetis*.

Given that a general drift response exists for Ephemeroptera to acids and metals, we used the ephemeropteran *Cinygmula* in this study as it was easy to find and was abundant in streams surrounding the affected reaches of Haggarty Creek. We designed drift chambers so *Cinygmula* would be easy to load and examine for drift; chambers also excluded predators. Chambers were used during the day to avoid a propensity for *Cinygmula* to drift at night, and we exposed *Cinygmula* for just 60 min to minimize drift due to a lack of food.

Results indicate that the drift behavior of *Cinygmula* is sensitive to the aqueous copper concentrations in Haggarty Creek. In particular, *Cinygmula* drift rate was significantly affected at all copper contaminated sites. Further, *Cinygmula* drift rate was sensitive enough to differ between the least contaminated Haggarty Creek site #3 and the more contaminated Haggarty Creek sites 1 and 2. We note that *Cinygmula* drift rate was not sensitive enough to differ between the two most heavily contaminated sites, suggesting that *Cinygmula* drift rate (60 min) may be at or near its maxima, for this system, at 78 ppb aqueous copper.

Currently, the USEPA standard for chronic continuous exposure to copper in freshwater systems is 9 ppb (USEPA 2005). That value is slightly less than the current value for acute exposure to copper in freshwater systems (13 ppb) (USEPA 2005). Given that *Cinygmula* exhibited a drift response to copper at 3 ppb, *Cinygmula* appears sensitive enough to indicate the presence of copper in Haggarty Creek at both the chronic and acute levels of contamination.

The results demonstrate that *Cinygmula* drift curves can be used in a simple *in situ* bioassay to determine whether aqueous copper concentrations in contaminated reaches of Haggarty Creek differ functionally from aqueous copper concentrations that occur in uncontaminated streams such as Bachelor Creek. Here we emphasize the functionality of such tests because aqueous copper concentrations are not being measured *per se*; rather, the effects of copper concentrations are being tested via drift behavior of *Cinygmula*. It may be possible to develop such tests for other point sources of pollution. Care must be taken, however, to both select an organism that is sensitive to specific types of pollution and will respond to pollution through drift behavior.

ACKNOWLEDGMENTS

We thank Joe Meyer, Director of the University of Wyoming Red Buttes Environmental Biology Laboratory, for allowing us use of that facility. This research was supported in part by grant no. USDA CSRELC 2000-01248.

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J. Ky. Acad. Sci. 67(2):109–116. 2006.

Phenotypic Distribution of *Nerodia erythrogaster* in Extreme Southeastern Illinois, Western Kentucky, and Adjacent Western Tennessee, USA

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ABSTRACT

To investigate anecdotal range distributions for *Nerodia erythrogaster,* a total of 130 adult specimens from southern Illinois, western Kentucky, and adjacent western Tennessee were scored for ventral color and dorsal pigment invasion onto the ventral scales. Analysis of these characters revealed that true *N. erythrogaster flavigaster* populations do not exist in Kentucky and also may be absent from Tennessee. The region is instead dominated by morphs intermediate between *N. e. flavigaster* and *N. e. neglecta.* Furthermore, the range of *N. e. neglecta* should be extended along the Ohio and Cumberland rivers.

KEY WORDS. *Nerodia, erythrogaster, flavigaster, neglecta, transversa,* phenotype, intergrade

INTRODUCTION

There are four recognized subspecies of *Nerodia erythrogaster*(Forster) in the United States: the yellow-bellied watersnake, *N. e. flavigaster* (Conant); red-bellied watersnake, *N. e. erythrogaster* (Forster); copper-bellied watersnake, *N. e. neglecta* (Conant); and blotched watersnake, *N. e. transversa* (Hallowell). (See Conant 1949 for detailed descriptions of each subspecies.) The distinctions between these subspecies are based primarily on three characters: ventral coloration, the presence of dorsal pigment encroaching onto the ventral scales, and the persistence of juvenile pattern into the adult stage. However, the range of ventral colors and degree of encroaching dorsal pigment for each subspecies has been defined arbitrarily (Brandon and Blanford 1995).

Nerodia erythrogaster is cryptic and occupies habitats that are not easily traversed by humans, which makes collecting an adequate sample size from a variety of locations difficult (Conant 1934; Ernst and Ernst 2003). This is perhaps why delineation of *N. erythrogaster* subspecies ranges have been based mostly on the analysis of preserved specimens. This is problematic because the colors of *Nerodia erythrogaster* fade in preservative (Conant 1949). Red and yellow pigments on the bellies of *N. erythrogaster* often completely disappear in a relatively short period of time, and even dark dorsal colors are affected to some degree. The geographic distribution of *N. erythrogaster* is further confounded because many early museum specimens were miscatalogued as *Nerodia sipedon* (L.) (Gibbons and Dorcas 2004).

In 1997, due to habitat loss and degradation, *N. e. neglecta* was listed as endangered by the states of Indiana, Ohio, and Michigan, and all *N. e. neglecta* populations above the 40th parallel were listed as threatened by the U.S. Fish and Wildlife Service (Pruitt 1997). More southerly populations (below the 40th parallel) were conferred special protection by the states of Illinois and Kentucky (Kingsbury 1998). Populations of *Nerodia erythrogaster* in

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extreme western Kentucky and adjacent western Tennessee are currently recognized as *N. e. flavigaster,* and populations of extreme southeastern Illinois are recognized as *N. e. neglecta* (Conant and Collins 1998). However, populations in Kentucky and Tennessee located west of the Tennessee river have received only anecdotal analysis (but see Bufalino 1999). The arbitrary nature of *Nerodia erythrogaster* taxonomy and the conservation issues surrounding *N. e. neglecta* warrant a thorough investigation of *N. erythrogaster* throughout its range. Herein, we present a quantitative description of the phenotypic variation exhibited by *N. erythrogaster* from the Ohio and Mississippi river systems of southern Illinois, western Kentucky and adjacent western Tennessee, and we provide an updated range map of the study area with insight on the consistency of separating specimens into phenotypic classes (i.e., subspecies) using Conant's (1949) diagnostic characters.

MATERIALS AND METHODS

Study Area and Sampling Methods

The study was conducted in portions of southern Illinois, the Jackson Purchase area of extreme western Kentucky (between the Mississippi and Tennessee Rivers), portions of Land Between The Lakes (LBL) National Recreation Area, and throughout adjacent western Tennessee (Figure 1). We captured 130 adult specimens (minimum SVL 480 mm; average SVL 792.77 ± 11.51 SE) by hand and between March 2002 and June 2004 and analyzed data from a minimum of 10 specimens from each of 11 sample sites. In order to avoid problems with color fading in preserved specimens, only living and recently killed specimens were analyzed. Live specimens were released unharmed at their site of capture.

Field Observations

In the field, snakes were classified as *flavigaster, neglecta,* or *neglecta* × *flavigaster* (specimens not falling within the acceptable range of variation for either *N. e. neglecta* or *N. e. flavigaster*) based on observations of the diagnostic characters described by Conant (1949). The anterior-most 10 belly scales (posterior to the chin), the 10 belly scales at midbody, and the 10 posterior-most belly scales (anterior to the anal plate) of each snake were scored with a Pantone Graphic Arts Color Key® (Pantone Inc., Carlstadt, New Jersey, USA) under natural lighting by holding the color tabs next to the darkest area of the snake's belly scales and selecting the corresponding tab. The proportion (%) of red hue present in the color score was recorded from lightest (yellow) to darkest (dark red). The color-scored belly segments also were digitally photographed on a neutral gray background under natural lighting.

Image Analysis

Photographs of the color-scored belly segments taken in the field were downloaded into Microsoft Photo Editor® (Microsoft Corporation, Mountain View, California, U.S.A.) for image analysis. The four best (free of scars and obscurities) belly scales of each 10-scale photograph were cropped and enlarged to 400× actual size. The total area (%) (in pixels) of the dorsal pigment present on each belly scale was determined, and the mean of the four-scale segment was recorded and used (along with the ventral color scores) in quantifying the differences among specimens assigned to phenotypic groups in accordance with Conant's (1949) qualitative methods (SAS Institute Inc., Cary, North Carolina, U.S.A.).

RESULTS

Phenotypic Variation

Adult *Nerodia erythrogaster* specimens from this region exhibited subtle variations in dorsal pattern prominence and ground color. Dorsal patterns ranged from inconspicuous to a slight remnant of the juvenile pattern. Dorsal ground colors ranged from chocolate brown to olive/gray and from gray to black. Because of the high variability and absence of an observable color gradient in the dorsal phenotypes (e.g., similar to the yellow to red gradient on the venter), we focused our efforts on the belly scales. Specimens assigned to the *neglecta* group (as defined by Conant 1949) exhibited ventral phenotypes that ranged from light orange to fire red-orange with dorsal pigment invasion that ranged from moderate/heavy to profuse (almost melanistic). Specimens classified as *flavigaster* exhibited ventral phenotypes that ranged from pale yellow to a yellow/light orange mixture with little to no dorsal pigment invasion along the anterior

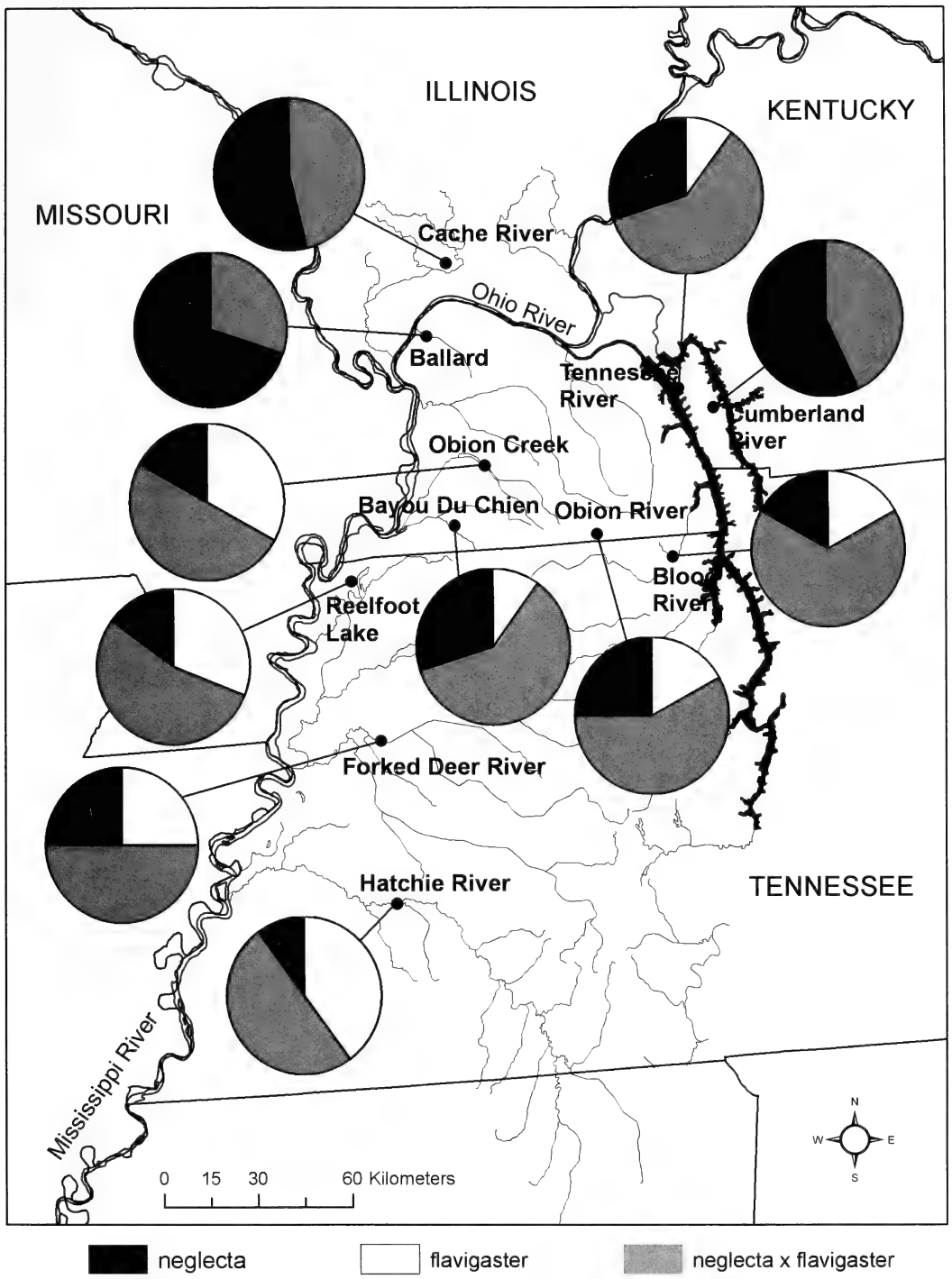

Figure 1. Study area with drainages and points indicating collection sites as well as phenotypic ratios of the populations sampled based on qualitative observations of Conant's (1949) ventral diagnostic characters.

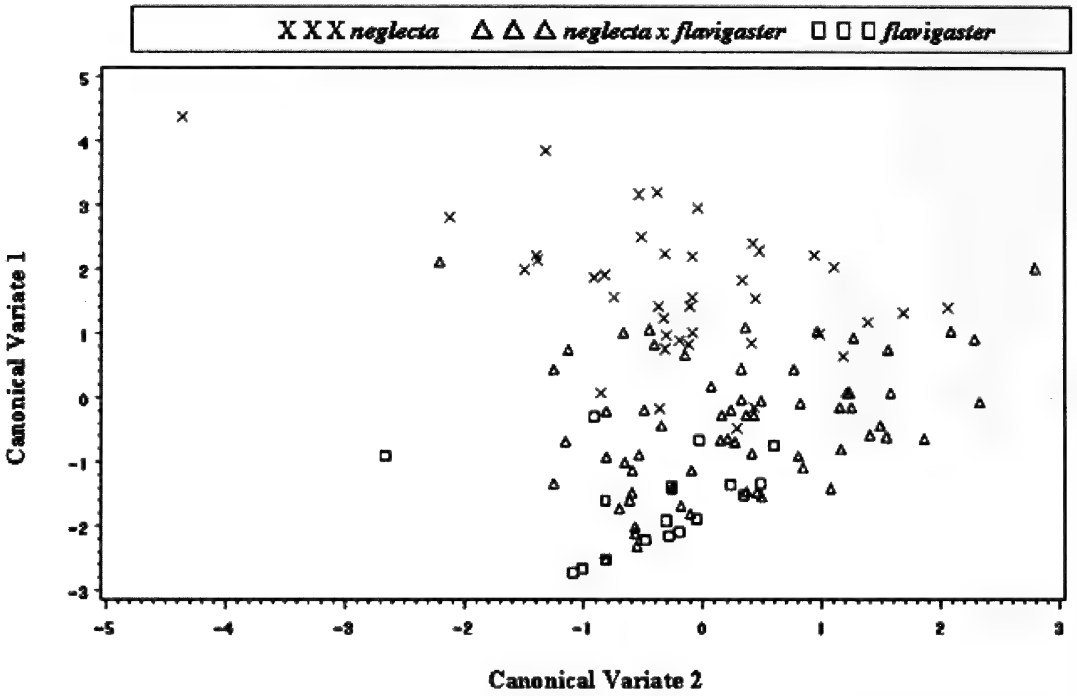

Figure 2. Plot of canonical variates created from ventral-scale data collected from each specimen illustrating the overlapping (or confusion) that can result when specimens from regions with broad phenotypic variation are assigned to phenotype groups (e.g., subspecies) using Conant's (1949) qualitative methods.

scale margin. Specimens classified as *neglecta* × *flavigaster* intermediates exhibited four general ventral phenotypes: 1) medium orange to red with little or no invading dorsal pigment (similar to *N. e. erythrogaster*), 2) a noticeably redder stripe running through the center of otherwise yellow scales, 3) the anterior half of each belly scale orange and the posterior half yellow, and 4) yellow and heavily mottled with dorsal pigment.

Phenotypic Classifications

Tukey's studentized range (HSD) (α = 0.05) revealed that phenotype groups were most strongly differentiated with mid-ventral observations: 1) all three groups had significantly different ($F_{2,130}$ = 15.60, $P < 0.001$) amounts (%) of red hue: *flavigaster* = 1.31 ± 0.23 SE; *neglecta* × *flavigaster* = 7.63 ± 0.95 SE; *neglecta* = 11.26 ± 1.01 SE and 2) all three groups had significantly different ($F_{2,130}$ = 78.06, $P < 0.001$) areas (%) of dorsal pigment invading into the belly scales: *flavigaster* = 6.93 ± 0.81 SE; *neglecta* × *flavigaster* = 18.13 ± 0.64 SE; *neglecta* = 39.16 ± 0.93 SE.

Discriminant and Canonical Discriminant Analysis

Discriminant analysis was used to determine the probability of misclassification using ventral color and the total area of dorsal pigment invading onto the ventral scales. The probability of misclassification was (23.44%) with eight *flavigaster* and eight *neglecta* misclassified as *neglecta* × *flavigaster*, seven *neglecta* × *flavigaster* misclassified as *flavigaster*, and seven more misclassified as *neglecta*. However, there were no instances where a *flavigaster* and/or a *neglecta* were mistaken for one another. Canonical discriminant analysis was used to create canonical variates based on the ventral data collected from each specimen to generate a plot that visually characterizes the overlap (or confusion) that occurs when separating *N. erythrogaster* into subspecies using qualitative observations (Figure 2).

Phenotypic Distribution

The ventral characters associated with *neglecta* (greater red coloration and dorsal pig-

ment invasion) reached their greatest values along the Ohio River and east of the Tennessee River. A transition occured through most of the Jackson Purchase and adjacent western Tennessee as these values became smaller. The ventral characters reached their lowest values in southwest Tennessee where the characters became more associated with the *flavigaster* phenotype (yellow coloration with little dorsal pigment invasion) (Figure 3). The phenotypic ratios at each sample site reflected a similar trend (Figure 1).

Intergradation Zone Delineation

Based on qualitative observations of specimens collected during this study and supported by population means of the ventral diagnostic characters described by Conant (1949), there was evidence of intergradation in all drainages but the Cumberland, Ohio, and Cache rivers. Intergradation may be occurring in these drainages as well, but no *flavigaster* phenotypes were observed, and the intermediates were visually much closer to *neglecta.* The findings suggested that the intergradation zone began south of the confluence of the Mississippi and Ohio rivers and extended east to the Tennessee River side of LBL, running at least as far south as the Hatchie River Bottoms of southwest Tennessee (Figure 4).

DISCUSSION

Nerodia erythrogaster populations of extreme western Kentucky and adjacent Tennessee exhibit a variety of phenotypes including individuals that are typical of all four United States subspecies. Within the study area, *N. e. neglecta* influence is greatest in the northern and eastern portions along the Ohio and Cumberland rivers. *N. e. neglecta* influence then gradually gives way to *N. e. flavigaster* influence in southwest Tennessee along the Mississippi River. The dominant phenotype class in the region, however, is a highly variable *neglecta* × *flavigaster* intergrade.

Brandon and Blanford (1995) suggested that a *neglecta* population will contain individuals typical of that subspecies as well as questionable forms that still bear a resemblance to *neglecta.* If so, the range of *N. e. neglecta* should be extended through the Ohio River (down to Ballard County, Kentucky) and through the northern half of the Cumberland River Basin (upstream to Trigg County, Kentucky). Furthermore, there is a large zone of intergradation south of the confluence of the Mississippi and Ohio rivers in the Jackson Purchase area of western Kentucky, east to the Tennessee River, and south to below the Hatchie River bottoms of southwest Tennessee. Thus, true *flavigaster* populations (populations consisting of individuals that only exhibit phenotypes typical of *flavigaster* or intermediate forms that favor *flavigaster*) do not occur in the state of Kentucky. Likewise, they might not occur in the state of Tennessee because the southward extent of the *neglecta* phenotype west of the Tennessee River (in some individuals) extends at least to the Hatchie River drainage and may reach as far south as northern Mississippi.

This study suggests that Conant's (1949) ventral diagnostic characters are reliable when separating specimens that are typical of *N. e. flavigaster* and *N. e. neglecta* from one another, but they lose considerable precision in regions or populations with broad phenotypic variation. Such populations can exhibit phenotypes other than those expected from a simple melding of phenotypes typical of an intergrade zone between two subspecies. For instance, in the field, 23 specimens indistinguishable from *N. e. erythrogaster* were observed, but for the needs of this study were classified as *neglecta* × *flavigaster* because the closest reported range of the red-bellied water snake subspecies (Conant and Collins 1998) is in rather distant southeastern Alabama.

With respect to *N. e. neglecta* conservation, the *Nerodia erythrogaster* populations of the Midwestern U.S.A. above the 40th parallel (regardless of subspecies) are threatened with extirpation from anthropomorphic activities and warrant each state's endangered status. *Nerodia erythrogaster* (including the *N. e. neglecta* phenotype) was well represented in the aquatic habitats investigated during this study, albeit, habitat degradation and fragmentation continue to be a concern for this or any wetland species occurring in the region. Thus, the protection currently provided by these state governments seems appropriate. However, our findings of *N. e. erythrogaster* phenotypes in western Kentucky and Tennessee along with Christiansen and Leclere's (2002) report of an *N. e. neglecta* population in Iowa

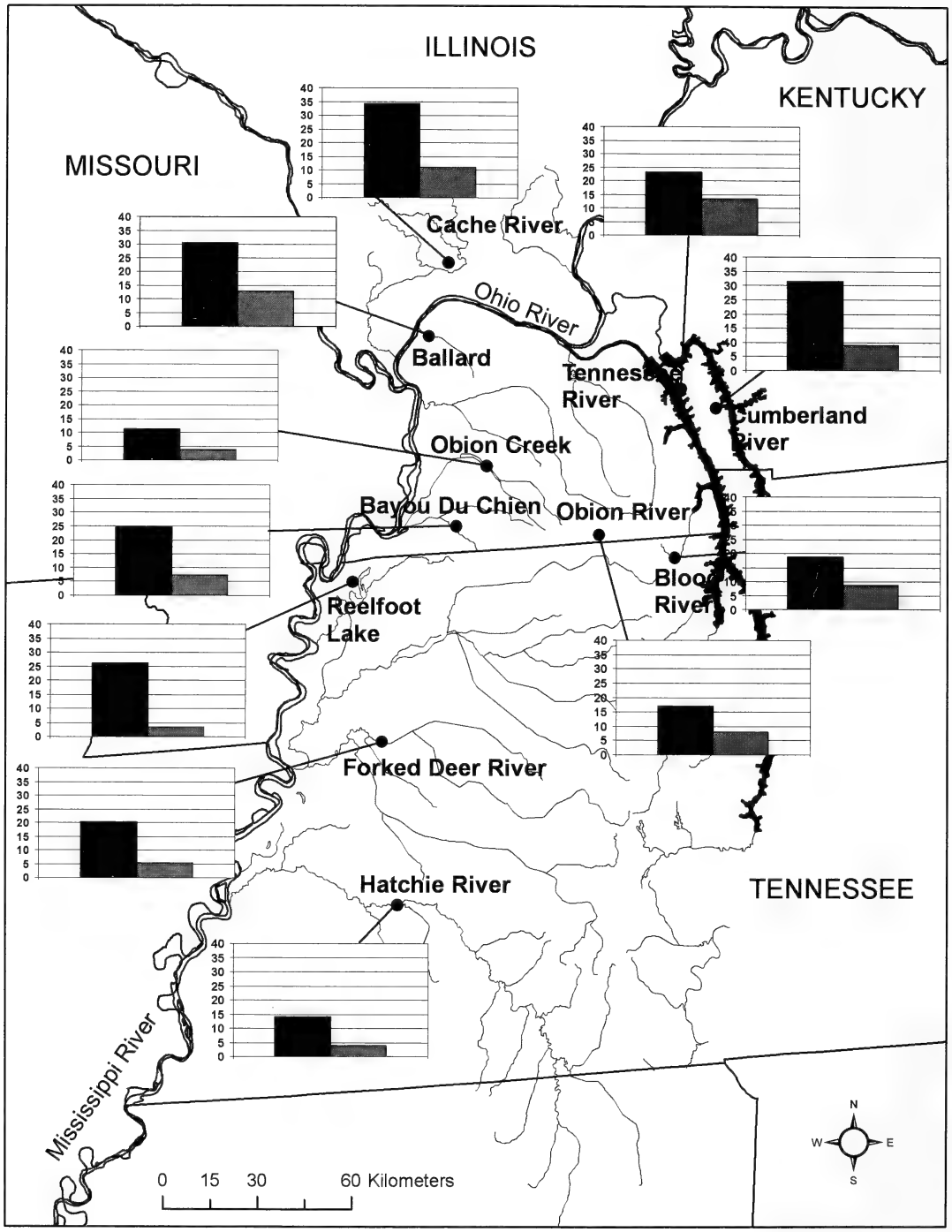

Figure 3. Mean proportion of red (gray bars), and the mean proportion of the ventral scales invaded by dorsal pigment (black bars) at the mid-ventral region for each sample population.

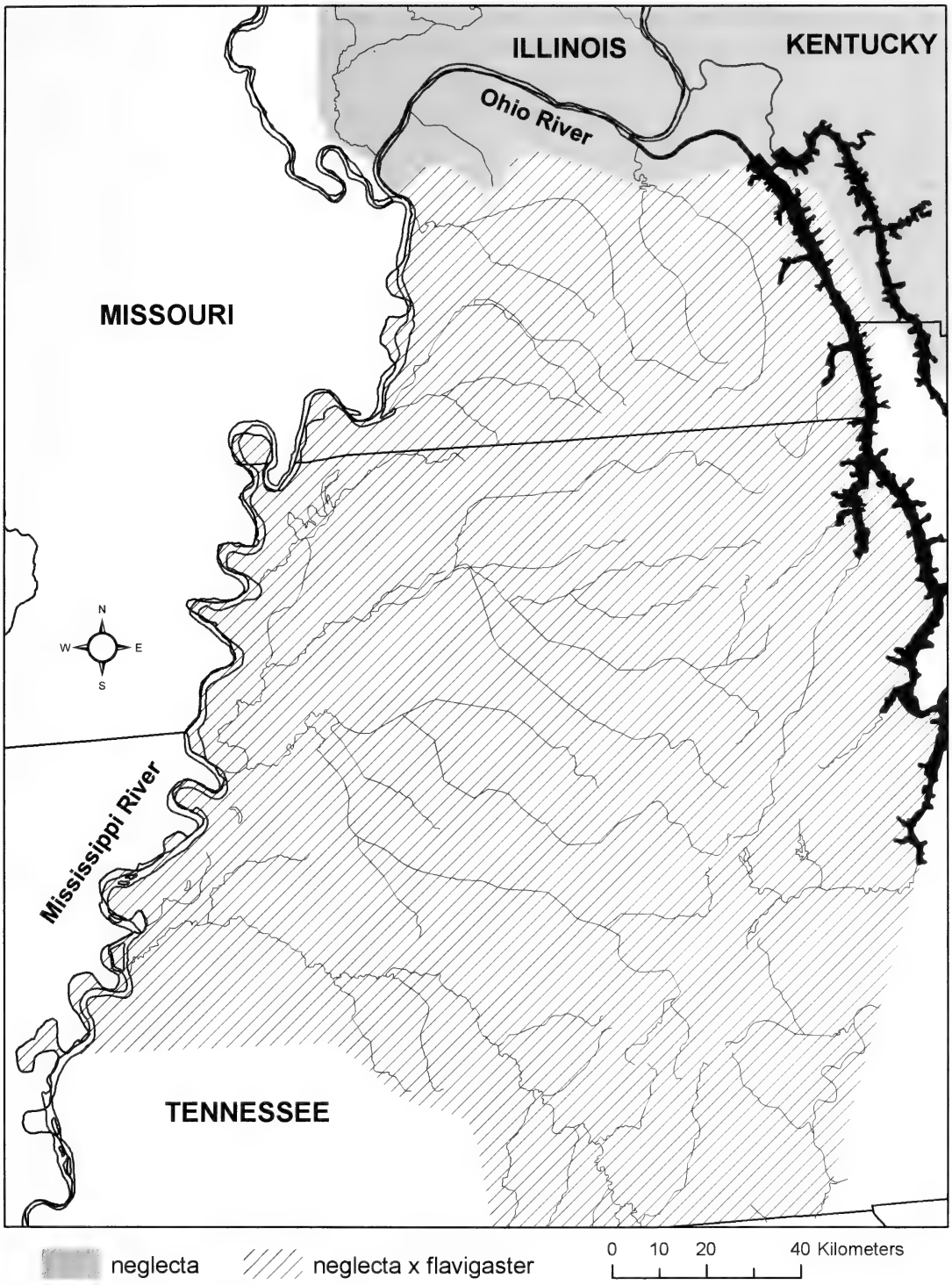

Figure 4. Revised *N. erythrogaster* range map for the study area. Only regions investigated during this study are delineated. Although perimeter areas such as southeast Missouri are visible in the figure, they were not investigated so they have been left idle.

bring into question the validity of subspecies distinctions in *Nerodia erythrogaster* altogether.

ACKNOWLEDGMENTS

All institutional animal care guidelines were complied with, and all required state and federal permits were obtained during this study. We wish to thank C. Crockett for field-contributions, C. Mecklin for statistical help, M. Busby for assistance with geographic images, H. Whiteman, L. Duobinis-Gray and P. Sikkel for thoughtful insights, and M. B. Zimmerer for extensive proofreading. We also are indebted to the Florida Fish and Wildlife Conservation Commission (FWC), Murray State University's Hancock Biological Station, and to R. Kipley of the U.S. Fish and Wildlife Service. This study would not have been possible without the permission of the U.S. Forest Service, the U.S. Fish and Wildlife Service, Kentucky Department of Fish and Wildlife Resources, Kentucky State Nature Preserves Commission, Tennessee Wildlife Resources Agency, and several private landowners.

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J. Ky. Acad. Sci. 67(2):117–124. 2006.

Inhibition of *Pyricularia oryzae*, the Gray Leaf Spot Pathogen of Perennial Ryegrass (*Lolium perenne*), by AH010, a Novel Fungicidal Material

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ABSTRACT

Mycelial growth of *Pyricularia oryzae* on amended potato-dextrose agar was inhibited to 50% of controls by ≥1250 ppm AH010 (40% benzalkonium chloride, 60% urea) and almost completely prevented by 10,000 ppm AH010. *P. oryzae* spore germination was completely inhibited by ≥18.6 ppm AH010 in sterile deionized water, and inhibition at 18.6 and 46.5 ppm AH010 was abolished by potato-dextrose broth. Sub-inhibitory concentrations of salicylic acid (SA; 50, 75, and 100 μM) negated the delaying effect of 9.3 ppm AH010 on spore germination. Maximal protection of perennial ryegrass plants from gray leaf spot disease (70–80% symptom reduction) was obtained with 2000 ppm foliar-applied AH010. Foliar-applied SA (2 mM) provided modest disease suppression that was dependent upon time of application and light regime. However, 2 mM SA antagonized disease suppression by AH010. Phytotoxicity of AH010 to *L. perenne* was observed, and this was conditioned by AH010 concentration and light regime.

KEY WORDS: *Pyricularia oryzae, Lolium perenne,* benzalkonium chloride, salicylic acid, light

INTRODUCTION

At present most American farmers protect their crop plants from diseases caused by fungal pathogens with synthetic fungicides that are thought to pose threats to human health and the environment. The utility of many of these fungicides also can be limited by the tendency of fungi to develop genetically transmissible resistance that renders these chemicals less effective, or ineffective in some cases. For these reasons, alternative means of plant disease suppression are under intensive investigation. A novel fungicidal material, AH010, is under development by United Promotions Incorporated (UPI, Atlanta,GA) as a possible replacement for standard fungicides. The active ingredient of AH010 is alkylbenzyldimethylammonium chloride (benzalkonium chloride), a quaternary ammonium compound that has been widely used as a surface disinfectant, primarily for the elimination of harmful bacteria in medical and food processing facilities. AH010 contains 40% benzalkonium chloride by weight, with 60% urea added as a stabilizer. AH010 is non-carcinogenic and biodegradable and is currently registered with the EPA for use as a disinfectant and algaecide for greenhouse floors and benches (UPI product, Timsen®) and for the control of diseases of ornamental crops under greenhouse production (UPI product, PronTech®).

The primary purpose of this research was to evaluate the ability of AH010 to suppress the *in vitro* and *in planta* development of *Pyricularia oryzae* Cav., a fungus that is the causal agent of the gray leaf spot disease of perennial ryegrass (*Lolium perenne* L.). This disease can cause serious reductions in turfgrass quality. Cultural practices are alone insufficient to control the disease. Although new cultivars of *L. perenne* with substantial resistance to the pathogen have been developed recently, it will be many years before they are widely planted, and it remains necessary to employ fungicides to obtain satisfactory disease control on existing susceptible turf (Paul Vincelli, Department of Plant Pathology, University of Kentucky, pers. comm.). Several of the most effective fungicides for control of this disease are at risk for developing resistance in the pathogen (Vincelli 1999), and the development of *P. oryzae* strains resistant to strobilurin fungicides has resulted in the failure of these fungicides to control the disease in some instances (Vincelli and Dixon 2002). The potential for salicylic acid (SA), an inducer of heightened disease resistance in many plant species (Delaney et al. 1994), to interact with

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AH010 in an additive or synergistic manner with respect to the suppression of *P. oryzae* was also investigated. SA was previously shown to possess direct antifungal activity and to synergistically enhance the activity of diverse other antifungal agents (Strobel and Porter 2005). Although it has been most intensively studied in dicotyledonous species, exogenous SA has been reported to enhance the heat tolerance of Kentucky bluegrass (*Poa pratensis* L.; He et al. 2005), activate the expression of genes related to disease resistance in wheat (*Triticum aestivum* L; Gorlach et al.1996), and protect barley (*Hordeum vulgare* L.) from oxidative damage incited by paraquat (Ananieva et al. 2004).

MATERIALS AND METHODS

Chemicals and Fungal Culture Materials

Deionized water (DIW) was used in the preparation of culture media for maintenance and preparation of spore inoculum of *P. oryzae*. AH010 was supplied by UPI (2030 Powers Ferry Road SE, Suite 136, Atlanta GA 30339). Salicylic acid (SA) was of USP grade. The fungicide Daconil 2787 (12.9% active ingredient chlorothalonil, manufactured by ISK Biotech Corporation, distributed by Dragon Corporation, Roanoke, VA 24019) was obtained locally.

P. oryzae Source, Maintenance, and Inoculum Preparation

P. oryzae isolate KY96, obtained from Dr. Paul Vincelli, University of Kentucky Department of Plant Pathology, was originally isolated from diseased *L. perenne*. For studies of *in vitro* mycelial growth, the fungus was maintained by serial mycelial transfer on potato-dextrose agar (PDA) incubated at room temperature (20–22 °C) in the dark. PDA was prepared by the addition of 2% non-nutritive agar to potato-dextrose broth (PDB). For studies of chemical effects on *P. oryzae* spore germination *in vitro*, spore production was accomplished by maintenance (via serial streaking of spores) on one-tenth strength oatmeal agar amended with non-nutritive agar to yield 2% agar content (DOA), with incubation at room temperature under continuous fluorescent lighting. Typically, spores produced within 6–8 d of transfer were employed for these studies. To harvest spores, culture plates were flooded with 5 ml DIW, their surfaces scraped with a sterile transfer loop, and the resultant suspension filtered through autoclaved cheesecloth to remove hyphal fragments and conidiophores. Spore density in filtered suspensions was determined with a haemocytometer. For studies of chemical effects on disease development in *L. perenne* plants, spore inoculum of *P. oryzae* was prepared each time by inoculating DOA with KY96 mycelia and spores that had been stored at −20 °C on colonized filter paper discs after routine re-isolation from *L. perenne* for the purpose of maintaining pathogenicity. Although maintenance of fungal pathogenicity was not crucial to the *in vitro* studies, a subsequent bioassay on *L. perenne* plants revealed that *P. oryzae* had not lost pathogenicity as a result of serial transfer on DOA.

In Vitro Inhibition of *P. oryzae* Mycelial Growth by AH010

The effect of AH010 on mycelial growth of *P. oryzae* was evaluated in polystyrene Petri plates (15 × 100 mm) containing PDA amended after autoclaving (but while still molten) with aliquots of filter-sterilized stock solutions of AH010. Inoculum plugs (approximately 4–6 × 4–6 mm) were cut from the actively growing margins of fungal colonies on PDA and placed in the centers of amended PDA plates (4 replicate plates per treatment in each experiment). Colony diameters were measured 12–14 d after inoculation. PDA not amended with AH010 served as the experimental control. Experiment 1 (conducted twice) employed six final concentrations of AH010 in PDA: 0, 250, 625, 1250, 1875, and 2500 ppm of formulated product. Experiment 2 (conducted twice) employed higher final concentrations of AH010 in PDA (0, 2500, 3000, 4000, 5000, and 10,000 ppm).

In Vitro Inhibition of *P. oryzae* Spore Germination in Sterile Deionized Water Amended With AH010 and/or SA

These experiments were conducted to more closely simulate the natural conditions of low nutrient availability under which *P. oryzae* spores normally germinate to initiate infection on perennial ryegrass leaves. Polystyrene 24-well culture plates received sterile deionized water and appropriate aliquots of filter-steril-

ized AH010 and SA to yield a volume of 400 μL in each well (four replicate wells per AH010 concentration were used in each experiment). Wells were then inoculated with 30 μL aliquots of *P. oryzae* spore suspension (1–2 × 10^4 spores per ml), and germination was assessed microscopically 16–18 hr after inoculation and again at 3–5 d after inoculation to detect delayed germination of spores. The purpose of these experiments was to identify concentrations of chemicals that resulted in 100% inhibition of spore germination. Wells in which germination appeared to be completely inhibited were thoroughly examined to ensure that no germinated spores were present. In wells in which germination had occurred, several microscope fields, each containing 10–20 spores, were examined, and percent germination estimated. Experiment 3 (conducted twice) employed final concentrations of 0, 465, 930, 1860, 2790, and 3720 ppm AH010 in each of four replicate wells. Experiment 4 (conducted twice) employed final concentrations of 0, 4.65, 9.3, 18.6, 46.5, and 93.0 ppm AH010 in each of four replicate wells per treatment. Preliminary experiments were conducted in well plates to assess the sensitivity of *P. oryzae* spore germination to SA. SA concentrations of 0, 100, 200, and 300, or 0, 50, 75, and 100 μM were selected for experiments 5a and 5b, respectively, which evaluated the interactive effects of SA and AH010 (0, 0.93, 1.86, 4.65, 9.3, and 18.6 ppm) on spore germination. Experiments 5a and 5b were conducted once, with a total of two replicate wells per treatment combination in each experiment.

Inhibition by AH010 of Gray Leaf Spot Disease Caused by *P. oryzae* on *L. perenne*

The ability of AH010 alone or in combination with 2 mM SA to protect perennial ryegrass plants from gray leaf spot disease was investigated in a wick-culture system according to the materials and methods of Vincelli and Dixon (2002) except where noted below. Experiment 6 employed foliar applications of 0, 500, 1000, 2000, 3000, or 4000 ppm of AH010 and was conducted twice. Positive controls were treated with a commercial protectant fungicide, Daconil, at the concentration recommended for control of gray leaf spot under field conditions. One day before chemical treatment pots containing approximately 40–50 two-week old *L. perenne* plants were trimmed to a height of 4–5 cm to provide uniform plant size and to ensure proper fit during the three-day incubation period in moist chambers. Chemicals were applied by spraying foliage to the point of runoff. After air-drying for one hour, plants in four replicate pots per treatment were inoculated by spraying with a suspension of *P. oryzae* spores (2–3 × 10^5 spores per ml) and sealed in moist chambers maintained in dim light for 24 hr followed by 48 hr under the normal fluorescent-lighting regime. After removal from moist chambers, plants were incubated for an additional 3–4 d under the normal light regime to allow the disease to develop. For disease assessment plant material in each pot was removed by gathering it together and clipping with scissors approximately 5 mm above the growth-medium surface. Twenty individual plants per pot were drawn randomly from each aggregate sample, and the percent of plant leaf area bearing necrotic lesions incited by *P. oryzae* was estimated visually. These data were summed and then divided by 20 to derive an average percent disease value for each pot. In the first trial of the experiment, plants in two additional replicate pots per chemical treatment were sprayed with deionized water free of *L. perenne* spores and then incubated as described to determine whether any of the AH010 concentrations employed were phytotoxic to *L. perenne*.

Interactive Effects of AH010 and SA on Development of Gray Leaf Spot Disease

Methods described above were employed to conduct a preliminary dose-response study with SA to identify a suitable concentration for use in the interaction studies with AH010. Two to three replicate pots per treatment were sprayed with unbuffered aqueous solutions containing 0, 0.5, 1, or 2 mM SA. The interactive effects of AH010 and SA on gray leaf spot disease development were examined in experiment 7 (conducted twice) in which methods differed in several details from those described above. Experimental plants were sprayed with chemical solutions (DIW, SA (2 mM), AH010 (2000 ppm), or SA (2 mM) + AH010 (2000 ppm) either 1 or 24 hr before inoculation with *P. oryzae*. The purpose of the

Table 1. Influence of 250–2500 ppm AH010 on mycelial growth of *Pyricularia oryzae* on potato-dextrose agar. Data presented are mean colony diameters (mm), standard errors, and percent relative to controls, for four replicate Petri plates per treatment in each of two experimental trials.

	AH010 (ppm)					
	0	250	625	1250	1875	2500
Trial I	70.9	63.5	49.5	41.9	30.8	23.5
	0.33	0.50	0.64	0.77	0.60	0.61
	(100)	(81.6)	(69.8)	(59.1)	(43.4)	(33.1)
Trial II	70.4	61.4	45.5	38.8	26.5	20.8
	0.94	1.11	0.61	0.32	1.06	0.52
	(100)	(87.2)	(64.6)	(55.1)	(37.6)	(29.5)

Table 2. Influence of 2500–10,000 ppm AH010 on mycelial growth of *Pyricularia oryzae* on potato-dextrose agar. Data presented are mean colony diameters (mm), standard errors, and percent relative to controls, for four replicate Petri plates per treatment in each of two experimental trials.

	AH010 ($\times 10^3$ ppm)					
	0	2.5	3.0	4.0	5.0	10.0
Trial I	69.3	21.0	18.0	11.4	10.8	7.8
	0.48	0.48	0.20	0.13	0.14	0.97
	(100)	(30.3)	(26.0)	(16.5)	(15.6)	(4.3)
Trial II	78.8	23.4	20.4	13.0	11.4	5.9
	0.43	0.13	0.38	0.20	0.22	0.31
	(100)	(29.7)	(25.9)	(16.5)	(14.5)	(7.5)

24-h pretreatment was to allow sufficient time for plant tissues to respond (e.g., via activation of antipathogen defense mechanisms) to chemical(s) that may have been absorbed through the cuticle. Plants were trimmed immediately before inoculation with the pathogen, rather than 24 hr before inoculation, to minimize excessive localized leaf wetness associated with contact of elongating shoots with the plastic covers of moist chambers. Plants in both trials of the experiment were inoculated by spraying with a spore suspension containing 1×10^5 spores/ml and were inadvertently maintained in dim light during the entire 3-d period of incubation within moist chambers, rather than the 1-d period of dim light in the dose-response studies. Significant water-soaking of older leaf tissues of all plants treated with 2000 ppm AH010 (regardless of SA treatment) was observed upon removal of plants from the moist chambers. Water-soaked tissues developed a bleached appearance (ranging from very pale tan to white) upon subsequent incubation under light. In most instances, this apparent phytotoxicity was readily distinguishable from the gray-green to brown coloration associated with necrosis induced by *P. oryzae*. In cases where the distinction was unclear, affected tissues were rated as necrotic due to *P. oryzae* infection. Estimates of percent necrosis reflected the extent of necrosis attributed to *P. oryzae* relative to the total leaf area (including portions damaged by AH010). Following harvest of plant material for disease assessment, the clipped pots were returned to the standard lighting regime for an additional 10 d to allow regrowth of surviving plants to occur. Plant tissues in each pot were cut 7–10 mm above the growth-medium surface to minimize contamination with necrotic plant material and fresh weight (mg) determined.

RESULTS

In Vitro Inhibition of *P. oryzae* Mycelial Growth by AH010

AH010 inhibited the mycelial growth of *P. oryzae* in a dose-dependent manner (Table 1). In both trials, concentrations in excess of 1250 ppm AH010 were required to inhibit mycelial growth by 50% relative to controls not exposed to AH010, and none of the concentrations employed abolished mycelial growth. Higher concentrations of AH010 resulted in greater inhibition of *P. oryzae* mycelial growth (Table 2). The onset of *P. oryzae* mycelial growth was substantially delayed at 5000 and 10,000 ppm, and after 14 d mycelial growth on PDA amended with 5000 ppm AH010 extended only a short distance into the amended agar. Short felt-like mycelium developed on the surfaces of the original inoculum plugs in Petri plates containing PDA amended with 10,000 ppm AH010. Mycelia did not extend into the amended agar in these plates.

In Vitro Inhibition of *P. oryzae* Spore Germination in Sterile Deionized Water Amended With AH010 and/or SA

In both trials of experiment 3, approximately 90% of spores germinated within 16–18 hr after their introduction to control wells containing only sterile DIW. No spore germination was detected in either trial at 16–18 hr or after 3–5 d in wells amended with AH010

(465–3720 ppm; data not shown). The effects of lower concentrations of AH010 (4.65–93.0 ppm) on spore germination were examined in Experiment 4. At 16–18 hr after inoculation, approximately 90% germination was observed in wells that contained either sterile DIW or 4.65 ppm AH010, but the germ tubes of spores in the latter wells were stunted when compared with those of water controls. No spore germination was observed at 16–18 hr after inoculation in wells containing 9.3, 18.6, 46.5, or 93.0 ppm of formulated product. When plates were observed 3–5 d after introduction of spores to wells, delayed germination of *P. oryzae* spores was observed the 9.3 ppm concentration of AH010 (germ tubes were stunted), whereas no germination was observed in wells that contained 18.6, 46.5, or 93.0 ppm of AH010.

A preliminary dose-response study with SA indicated that a concentration of 100 μM SA had no apparent effect on spore germination (which was approximately 90%, as in the DIW controls), whereas spore germination was strongly inhibited by 200 μM SA (the 2–5% of spores that germinated had stunted germ tubes) and completely inhibited by 300 μM SA. In experiments 5a and 5b, germination at 16–18 hr was comparable to that of DIW controls (approximately 90%) in wells containing 0.93, 1.86, or 4.65 ppm AH010. Germination at 9.3 ppm AH010 was delayed by 12–24 hr and resulted in relatively stunted germ tubes, and germination rarely occurred in wells containing 18.6 ppm AH010. Little or no germination occurred in any wells amended to contain 200 or 300 μM SA. Inclusion of 50, 75, or 100 μM SA in wells containing 0, 0.93, or 1.86 ppm AH010 did not affect germination relative to that of DIW controls. Inclusion of 100 μM SA in wells containing 4.65 or 9.3 ppm AH010 diminished percent germination to approximately 70–80% of DIW controls. SA concentrations of 50, 75, and 100 μM overcame the delay in germination otherwise observed at 19.3 ppm AH010 (germination occurred within 8 hr rather than 24–36 hr), and 100 μM SA sometimes enabled delayed germination (55–75%) in the presence of 18.6 ppm AH010. Thus, sub-inhibitory concentrations of SA appeared to antagonize the inhibitory effects of 9.3 and 18.6 ppm AH010 on spore germination.

Table 3. Influence of AH010 on severity of gray leaf spot disease in perennial ryegrass (*L. perenne*) inoculated with *Pyricularia oryzae*. Data presented are mean necrotic leaf area, standard error, and percent disease relative to controls, of four replicate pots per treatment in each of two trials.

	AH010 ($\times 10^3$ ppm)						
	0	0.5	1.0	2.0	3.0	4.0	Daconil
Trial I	63.4	48.5	18.8	12.3	11.5	17.8	0.0
	3.3	4.3	1.4	0.8	0.3	2.7	0.0
	(100)	(76.5)	(29.7)	(19.4)	(18.1)	(28.1)	(0.0)
Trial II	66.6	34.6	19.8	18.4	18.3	20.6	0.0
	3.2	3.4	1.9	2.7	1.2	1.0	0.0
	(100)	(52.0)	(29.7)	(27.6)	(27.5)	(30.9)	(0.0)

Inhibition by AH010 of Gray Leaf Spot Disease caused by *P. oryzae* on *L. perenne*

In experiment 6, control plants (sprayed with DIW one hour before inoculation with *P. oryzae* spores) were severely diseased, with an average percent necrotic leaf area of 63.4 and 66.6 in trials I and II, respectively (Table 3). Substantial reductions in disease development were observed for all concentrations of AH010 employed in these trials. In trial I, 2000 and 3000 ppm AH010 provided 80% reduction in symptomatic leaf area, relative to controls. A lesser degree of disease suppression by 2000 and 3000 ppm AH010 was observed in trial II (72% reduction, relative to controls). Interestingly, the highest concentration of AH010 employed, 4000 ppm, tended to provide somewhat less disease control (relative to 2000 and 3000 ppm) in both trials I and II. Plants not inoculated with *P. oryzae* showed similar patterns of growth for AH010 concentrations of 0, 500, 1000, 2000, and 3000 ppm, whereas plants treated with 4000 ppm AH010 were moderately stunted and exhibited a white to light tan chlorosis at the tips of some leaf blades. Daconil, a conventional synthetic fungicide that is registered with the EPA for use in controlling gray leaf spot, completely prevented disease development and was not phytotoxic in these trials.

Interactive Effects of AH010 and SA on Disease Development and Plant Regrowth

Exogenous SA affected disease development in a dose-dependent manner. Percent necrotic leaf area (mean ± SE) for plants sprayed with SA 0 (DIW controls), 0.5, 1, or

Table 4. Influence of AH010, salicylic acid (SA), and the timing of their application on severity of gray leaf spot disease and regrowth of perennial ryegrass (*L. perenne*) inoculated with *Pyricularia oryzae*. Data presented are mean necrotic leaf area (percent) and regrowth (mg fresh weight), standard errors, and percent disease and regrowth relative to controls. Trials I and II employed four and two replicate pots per treatment, respectively. 1 hr and 24 hr denote the number of hours between application of chemicals and inoculation with *P. oryzae*.

	AH010 (0 ppm) SA (0 mM)		AH010 (2000 ppm) SA (0 mM)		AH010 (0 ppm) SA (2 mM)		AH010 (2000 ppm) SA (2 mM)	
	1 hr	24 hr	1 hr	24 hr	1 hr	24 hr	1 hr	24 hr
Trial I Disease	48.2	50.6	18.4	15.4	41.8	55.4	25.2	23.6
	2.7	3.4	0.9	2.6	3.4	4.0	2.2	2.6
	(100)	(105.0)	(38.2)	(32.0)	(86.7)	(114.9)	(52.3)	(49.0)
Trial I Regrowth	43.5	31.0	84.0	93.3	82.5	34.5	74.0	76.5
	9.5	8.4	11.6	8.5	10.0	6.8	14.0	14.8
	(100)	(71.3)	(193.1)	(214.5)	(189.7)	(79.3)	(170.1)	(175.9)
Trial II Disease	69.5	70.4	30.3	38.6	48.3	67.9	30.9	38.5
	1.2	4.4	10.0	3.2	0.0	4.9	7.4	11.0
	(100)	(101.3)	(43.6)	(55.5)	(69.5)	(97.7)	(44.5)	(55.4)
Trial II Regrowth	11.5	24.0	91.5	37.5	42.5	31.5	68.0	80.5
	5.5	7.0	0.5	7.5	6.5	9.5	3.0	23.5
	(100)	(208.7)	(795.7)	(326.1)	(369.6)	(273.9)	(591.3)	(700.0)

2 mM were 65.4 ± 4.2, 39.2 ± 3.4, 56.1 ± 4.7, and 22.2 ± 5.9, respectively. 2 mM SA was selected for use in interaction studies with AH010 (experiment 7). The interactive effects of AH010 and SA, and the timing of their application, on disease development and plant regrowth were complex (Table 4). Despite several differences in methodology between this experiment and experiment 6, disease development in water-sprayed controls was roughly comparable to that in experiment 6, ranging from 48.2 ± 2.7% to 70.4 ± 4.4% in experiment 7 as compared with 63.4 ± 3.3% to 66.6 ± 3.2% in experiment 6 (Table 3). Disease suppression by 2000 ppm AH010 ranged from 44–68%, relative to water controls. The timing of treatment applications (either 1 or 24 hr prior to inoculation with *P. oryzae* spores) had no significant effect on disease development in water controls, or in plants sprayed with 2000 ppm AH010. The effects of SA on disease development were significantly affected by the timing of SA application to plants. When SA was applied 1 hr before inoculation with *P. oryzae,* the chemical had a slight inhibitory effect on disease development in the first trial, and suppressed disease development by 30% in the second trial, relative to water controls. Application of SA 24 hr prior to inoculation with *P. oryzae* resulted in disease development comparable to that in water controls, and significantly more disease than observed in plants sprayed with SA 1 hr before inoculation with the pathogen. A consistent pattern in the relative regrowth of plants after clipping to remove fungus-inoculated tissues was observed for both trials, although the trials differed in absolute amounts of regrowth (measured as mg fresh weight). Regrowth was least in pots that had sustained the greatest disease development (water controls and plants sprayed with SA 24 hr before inoculation with *P. oryzae*) and greatest in pots that had sustained the least amount of disease (those treated with AH010 at either 1 or 24 hr before inoculation with *P. oryzae,* and those treated with SA 1 hr before inoculation with the fungus).

DISCUSSION

Mycelial growth of *P. oryzae* on AH010-amended PDA (a nutritive medium) was far less sensitive to AH010 than was spore germination in AH010-amended DIW (the ED_{50} was 1250–1875 ppm for mycelial growth on PDA and 5–10 ppm for spore germination in DIW). The mechanism(s) underlying the greater sensitivity of spores to AH010 in DIW remain to be determined. Preliminary experiments, however, revealed that *P. oryzae* spores were able to germinate in the presence of 50 and 200 ppm AH010 in half-strength PDB. Thus the presence of nutrients in PDA may have contributed to the lesser sensitivity of *P.*

oryzae towards AH010 observed in studies of mycelial growth as compared with spore germination. Because a hazy appearance developed when >500 ppm AH010 was combined with half-strength PDB or with PDA, it is also possible that direct physical interactions of AH010 with one or more components of these media may have reduced the availability of the substance to the fungus.

Antagonistic interactions (*sensu* Kosman and Cohen 1996) of SA with AH010 were observed *in vitro* (spore germination assays) and *in planta* (disease suppression studies). In contrast, SA was previously reported to synergistically enhance the *in vitro* activity of several other antifungal agents (Strobel and Porter 2005). The mechanism(s) underlying the antagonism of AH010 by SA are unknown. The SA concentrations employed did not appear to interact physically with AH010 (no haze formation was observed) and did not reduce the pH of AH010 solutions below that indicated by the manufacturer to be suitable for antifungal activity of AH010 (pH 3.0). SA has been reported to activate an efflux pump in the plasma membrane of *Burkholderia cepacia* that confers resistance to multiple antibiotics (Nair et al. 2004). Increased activity of similar efflux pumps has been implicated in the tolerance of diverse bacteria to benzalkonium chloride (Poole 2005) and the pathogenicity of rice blast isolates of *P. oryzae* (Urban et al. 1999). It remains to be determined whether activation of a similar efflux pump contributes to the increased benzalkonium chloride tolerance of perennial ryegrass isolates of *P. oryzae* afforded by SA and constituents of PDA and PDB.

Although 20 ppm AH010 completely inhibited germination of *P. oryzae* spores *in vitro*, 500–4000 ppm AH010 afforded only partial protection of perennial ryegrass seedlings from *P. oryzae*. The reasons for this discrepancy are unknown. The maximum degree of disease suppression afforded by AH010 (80%) was significantly less than that afforded by Daconil (100%), a commercial fungicide commonly employed to suppress the disease under field conditions. The extent of disease suppression by AH010 was not enhanced by applying the chemical 24 hr prior to fungal inoculation or by combining AH010 with SA, although insufficient light availability during the early part of the incubation period in those experiments may have contributed to this finding (Chandra-Shekara et al. 2005; Genoud et al. 2002; Molina et al. 1998). Further testing is required to evaluate the effects of light on the interactions of AH010, SA, and *P. oryzae* with perennial ryegrass seedlings in controlled environments, and to evaluate the efficacy of AH010 for suppression of gray leaf spot disease under field conditions.

ACKNOWLEDGMENTS

This work was supported by grants from United Promotions Inc. and the Bluegrass Community and Technical College (Innovation Grant, 2005-2006). The author thanks Dr. Paul Vincelli (Professor) and Edward Dixon (Research Support Specialist) for providing cultures of *Pyricularia oryzae*, technical assistance and guidance, and the use of laboratory and plant culture facilities in the Department of Plant Pathology, University of Kentucky, Lexington. Thanks also to Larry Porter, Instructional Specialist, at Bluegrass Community and Technical College, for editorial assistance.

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J. Ky. Acad. Sci. 67(2):125–127. 2006.

NOTES

Prey Remains Identified in River Otter, *Lontra canadensis* (Schreber), Latrines from Eastern Kentucky—Information concerning the historic range of river otters in Kentucky is limited, but they probably once inhabited every major waterway in the Commonwealth (Cramer 1995). Otters were extirpated from Kentucky by the early-to-mid 1900s by the combined influences of unregulated harvest, habitat destruction, pollution, and human encroachment (Cramer 1995). In 1991 the Kentucky Department of Fish and Wildlife Resources initiated a river otter restoration project with the goal of restoring self-sustaining populations of otters throughout suitable habitat in the eastern half of the state. A total of 359 river otters was released statewide, with 155 of the animals being released at various sites throughout the Eastern Coal Field Region of Kentucky (Cramer 1995).

To gain an insight into the predatory role river otters play in Kentucky's rivers and lakes, portions of the drainages associated with six river otter release sites in the Eastern Coal Fields physiographic region of Kentucky—i.e., Little Sandy River, Red River, Tygarts Creek, Paintsville Lake, Middle Fork of the Kentucky River, and the Little Sandy River—were surveyed between June and December 1998 in order to locate *L. canadensis* latrines. River otters establish marking sites called latrines. Latrines, also known as pulling-out places (Liers 1951), sprainting areas (Erlinge 1967), haul outs (Mowbray et al. 1976), and landings (Melquist and Hornocker 1983), are shoreline locations where otters leave the water to defecate, urinate, and/or scent mark. Fecal material (scat) left at latrines contain the indigested portions of prey items consumed by river otters, e.g., fish scales and crayfish exoskeletons (Poole 1954).

In this study, otter scats associated with a latrine site were combined into one sample, dried, broken apart, and a subsample consisting of approximately 10% of the entire sample randomly selected for analysis. Items were identified based on exoskeleton parts, bones, scales, and microscopic characteristics of hair. All food items were recorded and diet composition determined based on frequency of occurrence. Analysis of 162 otter latrine samples revealed crayfish, fish, mammals, and insects were present in 98%, 57%, 6%, and 2% of the samples, respectively. No other animal remains, i.e., bird, reptile, amphibian, were found.

Serfass et al. (1990) found that 93% of otter scats in northeastern Pennsylvania contained fish, while crayfish were found in 4% of the scats. In Montana, Greer (1955) reported fish were most prevalent in scats, being detected in 93% of the scats examined; however, no crayfish remains were found. In coastal lakes in northern California, Modafferi and Yocum (1980) found that starry flounder (*Platichthys stellatus* 70% occurrence) and crabs (*Cancer* sp. 51%) were the most prevalent prey items in otter scats. In central California, Grenfell (1974) concluded crayfish were the most important food item for otters, being found in 98% of the scats examined. The major prey items identified in river otter latrine sites in eastern Kentucky, i.e., crayfish and fish, seem to reflect the general dietary pattern of the species throughout its range.

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Blue Crabs, *Callinectes sapidus* Rathbun, in Tennessee River Reservoirs—Mainstem reservoirs provide ideal habitats for invasive species of both plants and animals (Thornton et al. 1990; Yurista et al. 2000) but only rarely is the leap made between saltwater and freshwater habitats. The blue crab is widely distributed along the Gulf and Atlantic coasts of North America. Although reproduction must occur in salt waters (salinity >20 ppt) and females occur primarily where salinity is high, males are often found in low salinity estuaries to freshwaters (Guillory et al. 2001). Gunter (1938) recorded blue crabs as far as 305 km upstream in the Atchafalaya River, Louisiana, and there are a number of other distinctly freshwater records (Odum 1953; Wurtz and Roback 1955; Gunter and Hall 1963). To date, there had been no authenticated records of blue crabs from the Tennessee River or elsewhere in the Ohio River Basin.

The first record was a capture by a fisherman in Wheeler Lake near the Wheeler Dam, 28 June 2006. The crab was a female measuring 170 mm across the carapace (tip

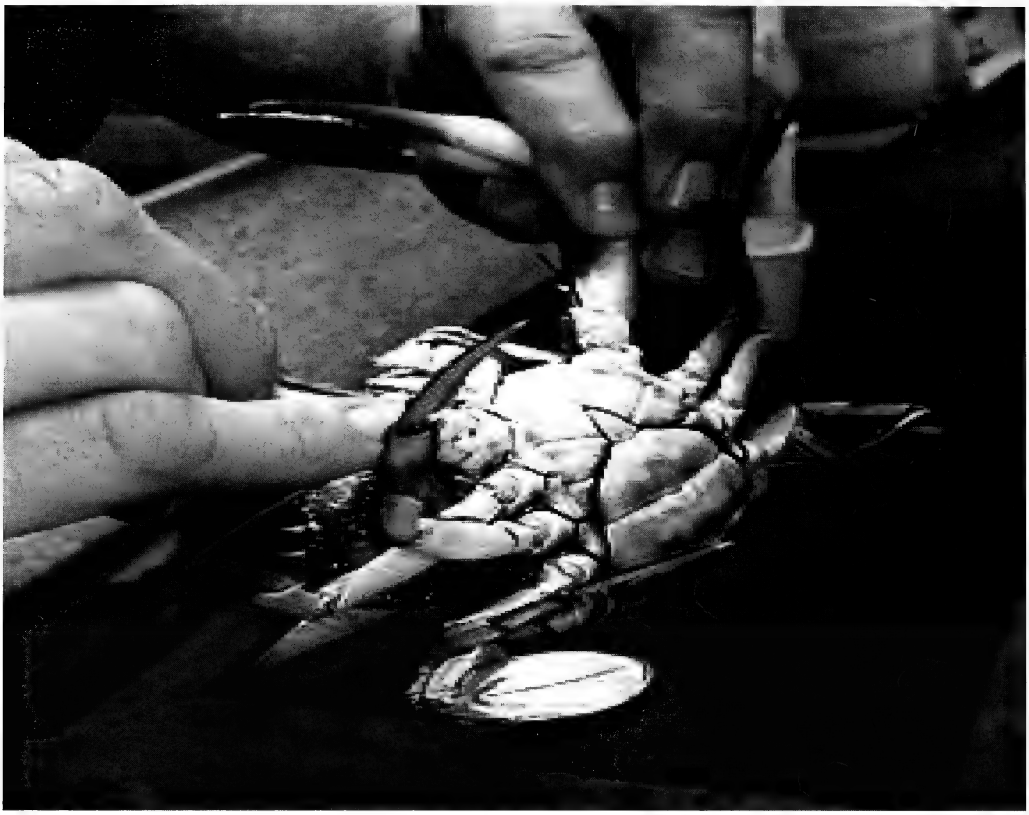

Figure 1. Female blue crab captured in Wheeler Lake, 28 June 2006. Photograph courtesy of Pat & Tim's Bait & Tackle at Fisherman's Resort, Joe Wheeler Dam.

of spine to tip of spine, Jivoff 1977) (Figure 1). It was kept alive at Pat & Tim's Bait & Tackle at Fisherman's Resort and lived 12 days in a fresh water minnow tank. The specimen was frozen after it died. A story on the crab written by Bryan Brasher appeared on 26 July 2006 at http://citizen.commercialappeal.com/theedge/2006/07/blue_crab_in_the_tennessee_riv.html.

On 21 August 2006, Mr. Jimmy Quillin of Big Sandy, Tennessee, captured a male blue crab in his commercial fishing net in Kentucky Lake (Tennessee River) near the Harmon Creek embayment, Benton County, Tennessee. The crab was captured in about 3 m (12 feet) of water in a weedy area (primarily *Ceratophyllum*). The crab was seemingly healthy (Figure 2) and was returned alive to Murray State University's Hancock Biological Station on Kentucky Lake. According to Mr. Quillin, it was the second blue crab he had caught, although the first had been much smaller. Alive, the crab weighed 513 g and measured 244 mm across the carapace (tip of spine to tip of spine). In comparison with other records (e.g., Guillory 2001), this was a very large specimen and may have been 4 or more years old (Tagatz 1968). The specimen has been preserved and archived in the collections of the Biological Station.

How and when the crabs arrived in the Tennessee River are open to speculation. If the crabs arrived as young adults, they must have been capable of surviving for several years. Larval and young crabs are known to have been dispersed accidentally in ballast water (Guillory et al. 2001) or by hitching rides on boats. Tennessee-Tombigbee Waterway provides direct shipping access from the Gulf of Mexico via the Mobile River to the middle reaches of the Tennessee River. While it is possible that males might survive that length of time, it seems doubtful that females would survive too long. Other potential explanations include escapees from crabs held for a later crab boil onboard the barge tugs and vacationing families releasing them into the reservoirs. It is safe to assume there aren't thousands of crabs in the river, but how is it that the few that are out there seem to be getting caught? We strongly suspect that the numbers of blue crabs in the Tennessee River system may be higher than one might expected.

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Figure 2. Male blue crab captured 21 August 2006 in a commercial fishing net in Kentucky Lake.

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The Kentucky Academy of Sciences and the JOURNAL also wish to give special thanks to **Varley E. Wiedeman** for his years of service as the Index Editor. It takes an especially organized person to do this job well. Ralph Thompson will take Varley's place on the Editorial Board.

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Compiled by Ralph L. Thompson

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